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*WITH 97 ILLUSTRATIONS*

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## PREFACE.

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BACTERIOLOGY is essentially a laboratory study. It is only by actual laboratory work that it can be taught in such a manner as to serve any useful purpose. It is also a subject of very general scientific interest. Courses in bacteriology are no longer confined to the medical schools, but are being introduced into colleges and agricultural and industrial schools. This volume has been prepared as a guide to the practical details of laboratory work. It is intended to present the subject in such a general way as to lay a broad foundation for later specialization in any branch of bacteriology. By a judicious selection the course can be made to conform to the requirements of medical, agricultural, or industrial students.

BROWN UNIVERSITY, August, 1901.

37436



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A LABORATORY COURSE  
IN  
BACTERIOLOGY



# A LABORATORY COURSE IN BACTERIOLOGY.

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## CHAPTER I.

### MICROSCOPIC EXAMINATION OF BACTERIA.

#### I. MANIPULATION OF THE MICROSCOPE. •

1. Examine mounted slides of bacteria with the low-power ( $\frac{2}{8}$  inch) and high-power ( $\frac{1}{8}$  inch) objectives and with different eyepieces.

2. Manipulate the condenser, diaphragm, and mirror, in order to ascertain what combination gives the best result.

3. (a) Affix the oil-immersion lens ( $\frac{1}{12}$  inch) to the microscope.

(b) Place a drop of immersion oil on the cover-glass.

(c) Bring the lens to a focus in the drop of oil.

(d) Again manipulate condenser, diaphragm, and mirror to determine what combination now is best.

#### II. MEASUREMENT OF BACTERIA.

The most accurate method of measurement is by photography;<sup>1</sup> but as this requires special appa-

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<sup>1</sup> See Appendix, page 163.



ratus, the following fairly accurate methods are recommended :

### 1. First Method.

(a) Place a micrometer eyepiece in the microscope.

(b) Examine the bacteria to be measured, and record their lengths in divisions of the eyepiece micrometer.

(c) Remove the slide of bacteria.

(d) Place a stage micrometer on the stage of the microscope, and determine the relation of the divisions of the eyepiece micrometer to the divisions of the stage micrometer.

(e) The length of the divisions of the stage micrometer is a fixed quantity (usually  $\frac{1}{100}$  mm.); therefore we have the equations :

Length of bacteria =  $x$  divisions of eyepiece micrometer ;

$x$  divisions of eyepiece micrometer =  $y$  divisions of stage micrometer ;

1 division of stage micrometer =  $\frac{1}{100}$  mm.

From these equations we can readily determine the length of the bacteria in millimeters. The unit of length for microscopic measurements is the thousandth part of a millimeter ; it is called a micron or micromillimeter, and is designated by the Greek letter  $\mu$ . Therefore in the above equations we can substitute for  $\frac{1}{100}$  mm.  $10 \mu$ , and write the result in so many  $\mu$ .

### 2. Second Method.

(a) Adjust the "camera lucida" to the microscope.

(b) Draw by means of the camera the exact size of the bacteria to be measured.

(c) Remove the slide of bacteria, and replace it with the stage micrometer.

(d) Over the figure of the bacteria now draw the scale of the stage micrometer, and read off directly the size of the bacteria in divisions of the stage micrometer.

### III. EXAMINATION OF LIVING BACTERIA.

#### I. Ordinary Examination.

(a) Place a drop of water on a clean slide.

(b) Pass the cotton plug of the tube containing the culture to be examined through the flame; ex-



FIG. 1.—Method of holding tubes during inoculation (McFarland).

tinguish the ignited cotton by blowing or pinching out the flame.

(c) Hold the test-tube containing the culture be-

#### 14 MICROSCOPIC EXAMINATION OF BACTERIA.

tween the thumb and finger of the left hand, allowing the lower end of the tube to rest on the back of the hand.

(d) Hold a straight platinum needle between the thumb and forefinger of the right hand, and sterilize it by heating red-hot. Allow it to cool.

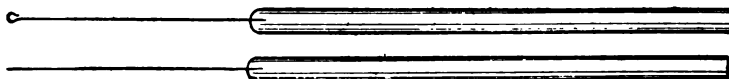


FIG. 2.—Platinum needles for transferring bacteria; made from No. 27 platinum wire inserted in glass rods.

(e) Grasp the cotton plug between the third and fourth finger of the right hand, and remove it; insert the platinum needle, and transfer an exceedingly minute portion of the culture of bacteria to the drop of water.

(f) Return the cotton plug to the tube, and sterilize the needle.<sup>1</sup>

(g) Place a clean<sup>2</sup> cover-glass over the drop of water, and examine with the  $\frac{1}{8}$  inch objective.

<sup>1</sup> The needle should invariably be heated before and after using. If this practice is not carefully followed, cultures will be contaminated, and perhaps pathogenic organisms spread about the room. It is well also to pass the handle of the needle through the flame before beginning work each day.

<sup>2</sup> Cover-glasses and slides may be cleaned in the following solution :

Potassium bichromate,	6 gm. ;
Concentrated sulphuric acid,	6 c.c. ;
Water,	100 c.c.

Wash in water and store in alcohol.

Or boil the cover-glasses in sulphuric acid, wash in

(h) Manipulate condenser, diaphragm, and mirror to determine the best adjustment for the examination of unstained bacteria.

**2. Examination in the "Hanging Drop."**

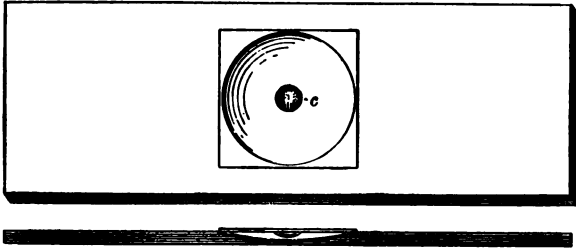


FIG. 3.—A "concave slide" with "hanging drop" (McFarland).

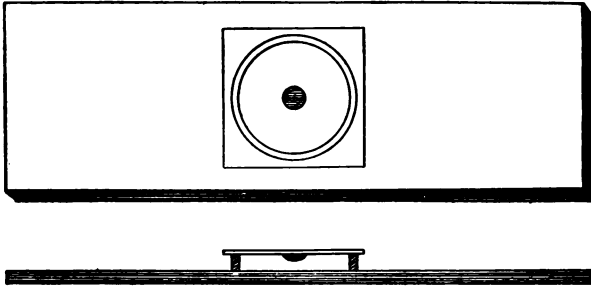


FIG. 4.—A slide with cell for hanging drop. The ring may be made of glass or of zylonite, and is cemented on the slide with Canada balsam.

(a) Paint a ring of vaselin around the hollow in water, and keep in alcohol; or wash in strong nitric acid for some time, rinse in water, and store in alcohol. Very often simply passing them several times through a Bunsen flame will clean them sufficiently.

a "concave slide;" or use a slide on which is cemented a small glass ring, and vaselin the top of the ring.

(*b*) On the centre of a clean cover-glass place a small drop of water.

(*c*) With a sterile platinum needle add to the drop of water a very small portion of the culture to be examined.

(*d*) Invert the slide over the cover-glass, so that the drop of water is covered by the concavity or is inside the glass ring, but does not touch the sides of either; press down so that the chamber is sealed tight by the vaselin.

(*e*) Invert carefully and examine.

The hanging-drop examination is for the purpose of determining the motility of bacteria or for watching their reproduction. The preparation may be kept for examination from day to day without loss by evaporation.

#### IV. EXAMINATION OF STAINED BACTERIA.

##### I. Ordinary Stains.

(*a*) Prepare a clean cover-glass.<sup>1</sup>

(*b*) Place a drop of water on the glass.<sup>2</sup>

(*c*) With a sterile platinum needle transfer a minute portion of a culture to the drop of water

---

<sup>1</sup> See footnote, page 14, for directions for cleaning covers.

<sup>2</sup> If the cultures are in bouillon or other fluid, it is often unnecessary to use the drop of water in spreading them on the cover-glass.

and spread uniformly over the surface of the cover-glass.<sup>1</sup>

(d) Allow the film to dry.

(e) When dry pass the cover-glass, smeared sur-



FIG. 5.—Cornet's cover-glass staining forceps.



FIG. 6.—Stewart's cover-glass forceps.



FIG. 7.—Kälteyer's cover-glass staining forceps.

face upward, three times through a Bunsen or alcohol flame at about the rate of the pendulum of a clock.

The heat coagulates the albuminous material

<sup>1</sup> The cover may be held, while staining, in one of the special forceps devised for the purpose.

around the bacteria and fixes them firmly to the glass.

(f) Place a drop of stain <sup>1</sup> on the cover-glass large enough to cover the film. Allow it to stain for from two to ten minutes; the length of time depends on the stain, the strength of the staining solution, and the kind of bacteria.<sup>2</sup>



FIG. 8.—Bottles for stains.

(g) Wash in water, dry the unsmeared side on filter-paper, mount, film side down, in a drop of water on a clean slide, and examine with the  $\frac{1}{8}$  inch objective.

---

<sup>1</sup> Almost any of the anilin stains may be employed. Gentian-violet, basic fuchsin, and methylene-blue are those most commonly used. For methods of preparing these staining solutions, see Appendix, page 170.

<sup>2</sup> It very rarely happens that bacteria are over-stained. But if such is the case, either a new film must be prepared or the stain drawn with weak acetic acid (1 : 1000), or the cover-glass swept through 1 per cent. sulphuric acid. If the film is not sufficiently stained, repeat the staining process.

(h) If the bacteria are evenly distributed<sup>1</sup> and properly stained, dry both sides thoroughly between filter-paper and mount, film side down, in a drop of Canada balsam. Label and preserve.

## 2. Gram's Stain.

The value of this method depends on the fact that the mycoprotein of certain bacteria forms with an anilin dye and an iodid a compound insoluble in alcohol. There are many bacteria in which such an insoluble compound is not formed, and this method consequently has considerable diagnostic value.

(a) To a drop of water on a clean cover-glass add a very small amount of a culture of *Bacillus subtilis*.

(b) To the same drop add a very small amount of a culture of *Bacillus vulgaris*, or any culture that does not stain by this method.

(c) Dry, fix, and stain for five minutes in anilin gentian-violet.<sup>2</sup>

(d') Wash in water.

(e) Treat with Gram's solution<sup>3</sup> for one minute.

<sup>1</sup> In a successful preparation the bacteria are evenly and not too thickly distributed over the surface. If too many bacteria are present, either a smaller amount of the culture must be used, or a little from the first cover-glass must be added to another drop of water on a second cover-glass, and so on until the proper dilution is reached.

<sup>2</sup> See Appendix, page 171.

<sup>3</sup> Gram's solution :

Iodin,	1 part ;
Potassium iodid,	2 parts ;
Distilled water,	300 parts.



(*f*) Wash in 95 per cent. alcohol until no more color comes away.

(*g*) Dry and contrast-stain in safranin<sup>1</sup> (thirty seconds), or Bismarck-brown<sup>2</sup> (two to three minutes), or eosin<sup>3</sup> (one minute).

(*h*) Wash, dry, and mount.

### 3. Staining Bacteria in Tissues.

#### (1) First Method.

(*a*) Harden the tissue in absolute alcohol or Zenker's fluid.<sup>4</sup>

(*b*) Dehydrate, embed, and section by the usual methods.

(*c*) Stain as directed for cover-glass preparations, but a little more deeply.

(*d*) Draw the color with dilute acetic acid (1 : 1000) until the bacteria alone are stained.

(*e*) Contrast-stain in eosin or any stain not requiring acid for differentiation.

(*f*) Clear and mount.

#### (2) Second Method.

Stain the sections by Gram's method as follows :

(*a*) Stain in anilin gentian-violet for five minutes.

(*b*) Wash in water.

(*c*) Treat with Gram's solution for one minute.

(*d*) Wash in 95 per cent. alcohol until no more color comes away.

---

<sup>1</sup> Stock solution is a 1 per cent. solution of safranin in equal parts of methylated spirit and water. For use, dilute with 5 parts of water.

<sup>2</sup> Saturated solution in equal parts of alcohol and water.

<sup>3</sup> Aqueous solution, 1 : 1000.

<sup>4</sup> See Appendix, page 173.

- (e) Wash in water.
- (f) Contrast-stain in an aqueous solution of eosin (1 : 1000) for one-half to one minute.
- (g) Wash in 60 per cent. alcohol for thirty seconds.
- (h) Wash in absolute alcohol for thirty seconds.
- (i) Clear in xylol and mount in balsam.
- (3) **Third Method.**
- (a) Stain in Kuhne's methylene-blue<sup>1</sup> for one-half to one hour, or in carbol-thionin-blue<sup>2</sup> for five minutes.
- (b) Wash in water.
- (c) Treat with 0.5 per cent. acetic acid till pale green.
- (d) Wash in water, 60 per cent. alcohol, and absolute alcohol, each for thirty seconds.
- (e) Contrast-stain as in above methods, clear, and mount.
- (4) **Fourth Method.**
- (a) Stain the dried preparations in a dilute aqueous solution of methylene-blue.
- (b) Wash in water and dry.
- (c) Stain in aqueous eosin solution (1 : 1000) for one to one and a half minutes.
- (d) Dehydrate, clear, and mount.

---

<sup>1</sup> See Appendix, page 171.

<sup>2</sup> See Appendix, page 171.

## CHAPTER II.

### MORPHOLOGY OF BACTERIA.

BACTERIA are minute, unicellular, vegetable organisms. They consist of a sharply defined mass of protoplasm which reacts to anilin stains very much like the nuclei of other cells, and outside of this a more or less well-developed envelope. They are classified according to their form into three main groups, the spherical cocci, the rod-shaped bacilli, and the curved or spiral spirilla.

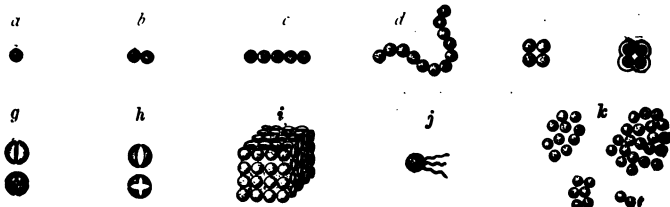


FIG. 9.—Diagram illustrating the morphology of the cocci : *a*, coccus or micrococcus ; *b*, diplococcus ; *c*, *d*, streptococci ; *e*, *f*, tetragenococci or merismopedia ; *g*, *h*, modes of division of cocci ; *i*, sarcina ; *j*, coccus with flagella ; *k*, staphylococci (McFarland).

#### I. Demonstration of Form.

(*a*) Make hanging-drop and stained preparations from cultures of cocci, bacilli, and spirilla.

(*b*) Examine with the  $\frac{1}{8}$  inch or with the oil-immersion lens.

In the hanging-drop preparations are the individual bacteria spherical, rod-shaped, or spiral? If rod-shaped, are the ends pointed, rounded, or square? Are the bacteria motile?<sup>1</sup> Are they single

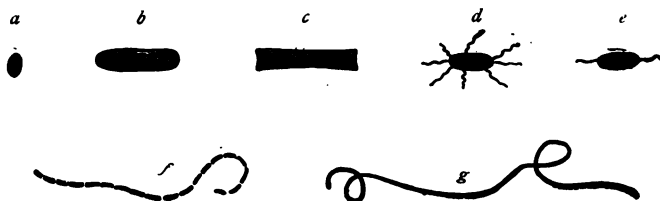


FIG. 10.—Diagram illustrating the morphology of the bacilli: *a*, *b*, *c*, various forms of bacilli; *d*, *e*, bacilli with flagella; *f*, chain of bacilli, individuals distinct; *g*, chain of bacilli, individuals not separated (McFarland).

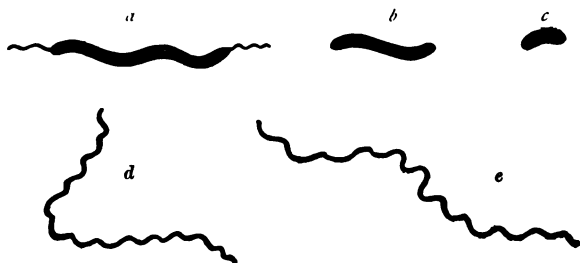


FIG. 11.—Diagram illustrating the morphology of the spirilla: *a*, *b*, *c*, spirilla; *d*, *e*, spirochæta.

or united in pairs, fours, irregular masses, or chains? Preserve the hanging-drop preparations for further study.

(*c*) Repeat these observations on the stained preparations.

<sup>1</sup> See § II., page 24.

Are the individuals stained uniformly or irregularly, deeply or faintly? In the rods are the ends more deeply stained than the centers (polar staining)?

(d) Measure the various kinds and make drawings of them.

## II. Demonstration of Motion.

(a) To a small drop of water on a cover-glass add a very little carmine or Bismarck-brown.



FIG. 12.—*Bacillus suipestifer*, showing flagella.

(b) Mount in the same manner as a hanging-drop preparation.

(c) Examine with the  $\frac{1}{8}$  inch objective.

Are the bits of pigment in motion? Do they change their position relative to one another, or do they dance about in one place? This is the so-called Brownian movement.

(d) Examine the hanging-drop preparations of the last section in reference to this movement.

Do they all show the Brownian movement? Are some actively swimming about, changing their position in relation to one another? All bacteria exhibit the Brownian movement, but certain ones are motile of themselves. They possess organs of locomotion or flagella, lash-like appendages, by the movements of which they propel themselves along. The flagella may be very numerous, extending from all sides of the cell, or they may be collected in a tuft at one end, or there may be a single one or a pair (peritrichous, lophotrichous, monotrichous, amphitrichous). The flagella may be demonstrated by appropriate methods of staining.

### III. Staining Flagella.

#### 1. Pitfield's Method.

(a) Prepare a mordant as follows :

Tannic acid (10 per cent. solution, filtered),	10 c. c. ;
Corrosive sublimate (saturated aqueous solution),	5 c. c. ;
Alum (saturated aqueous solution),	5 c. c. ;
Carbol-fuchsin <sup>1</sup>	5 c. c.

Allow to stand and draw off the clear fluid. The mordant will keep one or two weeks.

---

<sup>1</sup> See Appendix, page 172.

(b) Prepare a stain as follows :

Alum (saturated aqueous solution),	10 c. c.;
Gentian-violet (saturated alcoholic solution),	2 c. c.

The stain will keep two or three days.

(c) On an *absolutely* clean cover-glass<sup>1</sup> place a drop of distilled water.

(d) With a sterile platinum needle add to the drop of water, without stirring, a very small portion of an actively motile culture.

(e) Stand on a water-bath at 60° C. for one-half hour.

(f) Treat the film with the cold mordant for twenty-four hours, or with hot mordant (steaming, but not boiling) for three minutes.

(g) Wash thoroughly in running water and dry.

(h) Treat with the stain the same as directed for the mordant.

(i) Wash, dry, mount, and examine with the oil-immersion lens.

## 2. Modification of Pitfield's Method.

(a) Prepare solution A as follows :

Alum (saturated aqueous solution),	10 c. c.;
Gentian-violet (saturated alcoholic solution),	1 c. c.

---

<sup>1</sup> See footnote, page 14.

(b) Prepare solution B as follows :

Tannic acid,	1 gm.;
Distilled water,	10 c.c.

(c) Filter when cold, mix the two solutions, and use immediately.

(d) Prepare films as before.

(e) Treat with the above mixture, gently heating until it almost boils, then set aside for a minute.

(f) Wash, dry, and mount.



FIG. 13.—*Microspira comma*, showing the flagella;  $\times 1000$  (Günther).

### 3. Löffler's Method.

(a) Prepare a mordant as follows :

Tannic acid (20 per cent. solution), filtered,	10 c.c.;
Ferrous sulphate (cold, saturated solution), filtered,	5 c.c.;
Fuchsin (saturated alcoholic solution),	1 c.c.



• • • • • BACTERIA.

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the same.

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I have been told that the "counter-revolution" and "white" movement are going to take over the nation and that the "black" movement will be crushed.

for information, see

(g) Wash in water, dry, mount, and examine with oil-immersion lens.

**4. Modification of Löffler's Method.**

(a) Prepare a mordant as follows :

Tannic acid (20 per cent. solution),	10 c. c. ;
Ferrous sulphate (saturated solution),	10 c. c. ;
Logwood solution (1 gram boiled in 8 c.c. of water and filtered),	3-4 c. c.

(b) Prepare a stain as follows :

Anilin-water, <sup>1</sup>	100 c. c. ;
Sodium hydrate (1 per cent.),	1 c. c. ;
Methylene-violet or methylene-blue or fuchsin,	4-5 gm.
Filter.	

(c) Prepare films as before.

(d) Heat with the mordant until steam rises, and then move over flame for one minute.

(e) Wash in water, and if mordant does not disappear, in absolute alcohol.

(f) Dry and heat with the stain until steam rises, then leave in the warm stain one minute.

(g) Wash, dry, and mount.

---

<sup>1</sup> See Appendix, page 171.

### 5. Van Ermengem's Method.<sup>1</sup>

(a) Prepare solution A as follows :

Osmic acid (2 per cent. solution), 1 part ;

Tannic acid (10 to 25 per cent. solution), 2 parts.

To each 100 c.c. of the tannic acid add 4 or 5 drops of glacial acetic acid.



FIG. 15.—Bacterium Faschingii in blood ;  $\times 1000$  (Fränkel and Pfeiffer).

---

<sup>1</sup> Various modifications of Van Ermengem's method have been suggested. See *Centralbl. f. Bakt.*, Erste Abteilung, Bd. xxvii., Nos. 16-17, p. 597 ; and *Lancet*, vol. ii., No. 14, p. 874, 1898.

(b) Prepare solution B as follows :

Gallic acid,	5 gm. :
Tannic acid,	5 "
Fused acetate of sodium or	
potassium,	10 "
Distilled water,	350 c.c.

(c) Prepare films as before.

(d) Place in solution A for one-half hour : or, if the solution is warmed to 60° C., five minutes are sufficient.

(e) Wash thoroughly in water, then in alcohol, then again in water.

(f) Place for two minutes in solution of nitrate of silver (0.25-0.5 per cent.).

(g) Without washing, place in solution B for one and one-half to two minutes, using fresh solution for each preparation.

(h) Wash thoroughly in water, and examine in water. If the flagella are not sufficiently stained, begin again at (f).

Always change the silver nitrate solution as soon as any precipitate appears.

#### IV. Demonstration of Capsules.

In certain forms the envelopes surrounding the cells can be stained as follows :

##### 1. *Welch's Method.*<sup>1</sup>

(a) Prepare films without using water.

(b) Place in glacial acetic acid for a few seconds.

(c) Remove the acid with filter-paper.

<sup>1</sup> *Bulletin of the Johns Hopkins Hospital*, p. 128, December, 1892.

(*d*) Wash in anilin-water gentian-violet repeatedly until all the acid is removed.

(*e*) Wash in a  $\frac{1}{2}$  per cent. solution of sodium chlorid and examine in the same solution.

2. **Johne's Method.**

(*a*) Prepare films as usual.

(*b*) Warm in a 2 per cent. solution of gentian-violet until steam arises.



FIG. 16.—*Bacterium pneumoniae* of Friedländer, from the expectoration of a pneumonia patient;  $\times 1000$  (Fränkel and Pfeiffer).

(*c*) Wash in water.

(*d*) Place in 2 per cent. acetic acid for six to ten seconds.

(*e*) Wash in water.

(*f*) Dry and mount in balsam.

**3. Boni's Method.<sup>1</sup>**

(a) Prepare a solution as follows :

White of one egg ;

Glycerin, 50 c. c. ;

Formalin, 2 drops.

Shake together and filter.

(b) Prepare films of the organism to be studied, using the above solution in place of water.

(c) Spread very thin, and heat over flame until the glycerin is evaporated.

(d) Stain in Ziehl's carbol-fuchsin<sup>2</sup> for twenty to thirty seconds.

(e) Wash in water and dry with filter-paper.

(f) Contrast-stain in Löffler's methylene-blue<sup>3</sup> for four to six minutes.

(g) Wash, dry, and mount in balsam.

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<sup>1</sup> *Centralbl. für Bakteriologie*, Erste Ab., Band xxviii., No. 20, p. 705.

<sup>2</sup> See Appendix, page 172.

<sup>3</sup> See Appendix, page 171.

## CHAPTER III.

### REPRODUCTION OF BACTERIA.

THE common method of reproduction among the bacteria is by binary division. In some forms this is the only mode of reproduction known. Under favorable conditions an individual cell grows in length, a transverse constriction appears in the middle and gradually becomes deeper until two new cells are formed. Separation may be complete or there may be the formation of chains. In the spherical forms division may take place in one, two, or three planes, forming chains (streptococci), or groups of two (diplococci), irregular groups (staphylococci or micrococci), or cubical packets (sarcina). In the rod-shaped forms division takes place in but one plane, forming chains, pairs, or single individuals (Figs. 9 and 10).

Some bacteria, besides having the power of reproduction by division, form endogenous spores. These spores are developed from the plasma of the cell, and have a dense wall that protects them from injury by drying, enables them to withstand high temperatures, and also causes them to resist the action of stains.

#### I. Reproduction by Division.

(a) On a clean cover-glass place a large drop of bouillon.<sup>1</sup>

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<sup>1</sup> See page 50.

(b) Add to the bouillon a very small portion of a culture of *Bacillus subtilis*, or any chain-forming and spore-producing species.

(c) Mount as a hanging-drop preparation and place in the incubator.

(d) Examine at intervals of one-half hour.

Notice the elongation of the bacilli and the formation of chains.

(e) Inoculate a bouillon-tube as directed on page 60.

(f) Place in the incubator and make stained preparations at half-hour intervals.

Preserve both hanging-drop preparation and bouillon-tube for further examination.

## II. Reproduction by Spores.

1. After the formation of chains has taken place in the above cultures, continue the examination at intervals. The formation of spores within the rods will be observed; finally, the rods will disappear and nothing but spores remain.



FIG. 17.—Diagram illustrating sporulation: *a*, bacillus enclosing a small oval spore; *b*, drumstick bacillus, with the spore at the end; *c*, clostridium; *d*, free spores; *e*, and *f*, bacilli escaping from spores.

2. Make stained preparations from the bouillon culture when rods and spores within the rods and spores alone are all present. Stain as follows:



### III. Staining Spores.

#### (I) First Method.

- (a) Prepare films in the usual manner.
- (b) Stain in warm Ziehl's carbol-fuchsin<sup>1</sup> for twenty to thirty minutes, or in the same solution steaming, but not boiling, for five minutes.
- (c) Wash in water.
- (d) Dip in acid alcohol (70 per cent. alcohol, 97

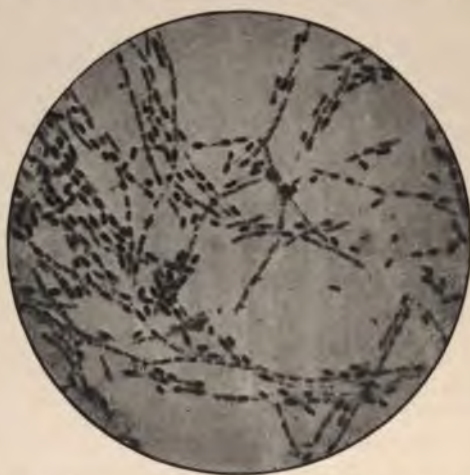


FIG. 18.—*Bacterium anthracis*, stained to show spores ;  
× 1000 (Fränkel and Pfeiffer).

c.c. ; hydrochloric acid, 3 c.c.) or in 1 per cent. sulphuric acid a few seconds and wash in water.

#### (e) Examine in water.

Spores should be red and rods unstained or faintly pink. If the spores are not sufficiently

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<sup>1</sup> See Appendix, page 172.

stained, place again in the fuchsin. If the rods are still stained, wash longer in the acid alcohol.

(*f*) Dry and pass three times through the flame.

(*g*) Stain in Löffler's methylene-blue<sup>1</sup> for three to four minutes, or in gentian-violet<sup>2</sup> one minute, or in saturated aqueous solution of methylene-blue for half a minute.

(*h*) Wash, dry, and mount.

Spores should be red, rods blue.

(2) **Second Method.**

(*a*) Prepare films in the usual manner.

(*b*) Place in chloroform for two minutes.

(*c*) Treat with 5 per cent. chromic acid for one minute. The acid acts on the membranes of the spores and permits the entrance of the stain.

(*d*) Wash thoroughly in water.

(*e*) Stain in hot Ziehl's carbol-fuchsin for three minutes.

(*f*) Without washing decolorize in 5 per cent. sulphuric acid.

(*g*) Wash in water and stain in Löffler's methylene-blue<sup>1</sup> for two minutes or longer.

(*h*) Wash, dry, and mount.

(3) **Third Method.**

(*a*) Suspend the spore-bearing bacteria in a normal salt solution<sup>3</sup> in a test-tube.

(*b*) Add an equal volume of Ziehl's carbol-fuchsin.

(*c*) Place the test-tube in boiling water for fifteen minutes.

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<sup>1</sup> See Appendix, page 171.

<sup>2</sup> See Appendix, page 171.

<sup>3</sup> See Appendix, page 173.

(d) Spread a loopful on a cover-glass; dry; fix.

(e) Decolorize in a  $1\frac{1}{2}$  per cent. (by volume) solution of hydrochloric acid in alcohol.

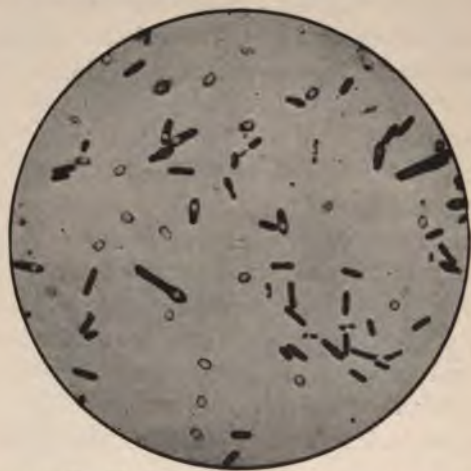


FIG. 19.—*Bacillus chauvæi*, showing spores;  $\times 1000$   
(Fränkel and Pfeiffer).

(f) Wash in water and contrast-stain in Löffler's methylene-blue.

(g) Wash, dry, and mount.

**(4) To Observe the Germination of Spores.**

(a) Make a hanging-drop preparation, using sterile bouillon and some of the spores from the above culture.

(b) Place on a warm stage and watch the growth of rods from the spores.

(c) Inoculate a sterile bouillon-tube with a large number of spores from the above culture.

(d) Make stained cover-glass preparations from this at intervals of one-half hour.

## CHAPTER IV.

### CLASSIFICATION OF BACTERIA.

THE position occupied by the bacteria in the vegetable world is shown by the table on page 40.

The Bacteria or Schizomycetes are classified into families according to their form, and again into genera according to certain other characters. The following classification is that proposed by Migula in his *System der Bakterien*, 1900 :

#### BACTERIA, SCHIZOMYCETES.

EUBACTERIA, cells contain no sulphur granules or bacterio-purpurin.

##### 1. Family COCCACEÆ, spherical forms.

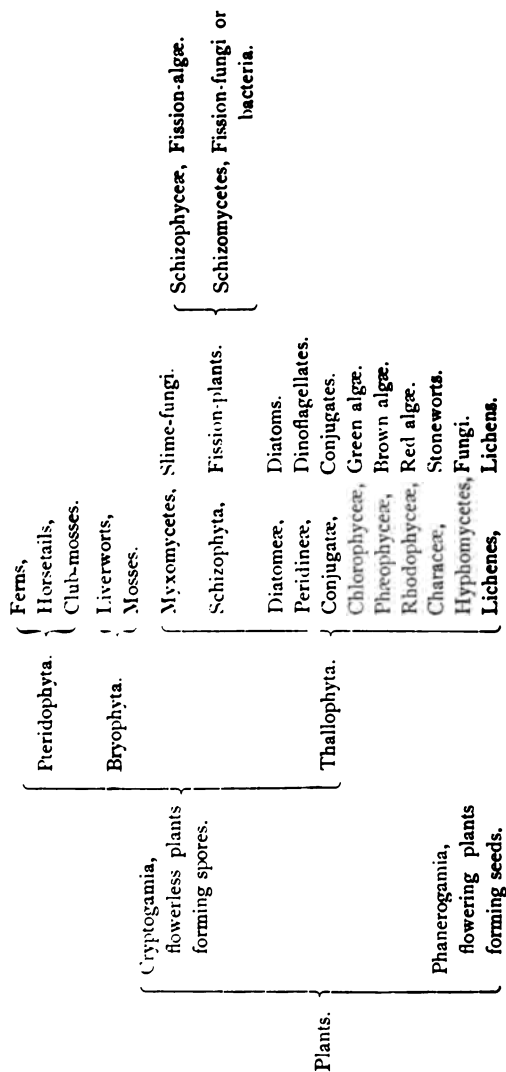
Genus :

1. *Streptococcus*, non-motile ; cells divide in one plane.
2. *Micrococcus*, non-motile ; cells divide in two planes.
3. *Sarcina*, non-motile ; cells divide in three planes.
4. *Planococcus*, motile ; cells divide in two planes.
5. *Planosarcina*, motile ; cells divide in three planes.

2. Family BACTERIACEÆ, straight, rod-shaped forms without envelope.

Genus :

1. *Bacterium*, non-motile.
2. *Bacillus*, motile ; flagella over whole surface.
3. *Pseudomonas*, motile ; flagella polar.



3. Family SPIRILLACEÆ, curved, rod-shaped forms without envelope.

Genus :

1. *Spirosoma*, non-motile ; cells rigid.
2. *Microspira*, motile ; one, rarely two or three, polar flagella.
3. *Spirillum*, motile ; polar tufts of flagella.
4. *Spirochæta*, cells flexile.

4. Family CHLAMYDOBACTERIACEÆ, cells with envelopes.

Genus :

1. *Chlamydothrix*, unbranched threads ; cell-division in one plane.
2. *Crenothrix*, unbranched threads ; cell-division in three planes ; sheath visible.
3. *Phragmidiothrix*, unbranched threads ; cell-division in three planes ; sheath scarcely visible.
4. *Sphærotilus*, branched threads.

THIOBACTERIA, cells contain sulphur granules or bacterio-purpurin ; red or violet color, never green.

1. Family BEGGIATOACEÆ, thread-forming, without bacterio-purpurin.

Genus :

1. *Thiotrix*, attached threads ; non-motile.
2. *Beggiatoa*, unattached threads ; motile.

2. Family RHODOBACTERIACEÆ, cells contain bacterio-purpurin and sulphur granules ; red or violet.

I. Subfamily THIOCAPSACEÆ, cells divide in three planes.

Genus :

1. *Thiocystis*.
2. *Thiocapsa*.
3. *Thiosarcina*.

II. Subfamily LAMPROCYSTACEÆ, cells divide first in three, then in two planes.

Genus :

1. *Lamprocystis*.

III. Subfamily THIOPEDIACEÆ, cells divide in two planes.

Genus :

1. *Thiopedia*.

IV. Subfamily AMEBOBACTERIACEÆ, cells divide in one plane.

Genus :

1. *Amebabacter*.
2. *Thiothece*.
3. *Thiodictyon*.
4. *Thiopolycoccus*.

V. Subfamily CHROMATIACEÆ.

Genus :

1. *Chromatium*.
2. *Rhabdochromatium*.
3. *Thiospirillum*.

## CHAPTER V.

### STERILIZATION.

STERILIZATION is the process of killing micro-organisms. A body is said to be sterile when all the bacteria in or upon it have been killed or removed. Sterilization is effected by the application of heat, by treating with certain chemicals, or, in the case of fluids, by filtration.

When spores are not present, bacteria are killed by exposure for twenty minutes to boiling water or steam. To destroy spores, however, a much longer exposure is necessary, in some cases several hours. Steam under a pressure of thirty pounds will give a temperature of  $120^{\circ}$  C.; this will kill all organisms and spores in fifteen minutes.

Dry heat is not so fatal to either bacteria or their spores as is moist heat or steam; therefore to sterilize by dry heat a higher temperature, sustained for a longer time, is necessary;  $150^{\circ}$  C. continued for one hour will kill all ordinary bacteria and their spores. Liquids and objects liable to be injured by dry heat can be sterilized only by steam.

The method of *discontinuous sterilization* is employed in sterilizing objects which would be injured by long exposure to a high temperature. The objects to be sterilized are first subjected to a temperature sufficiently high to destroy all bacteria not



in the spore condition, say  $100^{\circ}$  C. for twenty minutes. They are then allowed to cool for twenty-four hours, and again sterilized as before. This is repeated several times. The intermissions allow such spores as are present to develop into rods, and these are killed by the subsequent heating.

*Pasteurization* is the term applied to the partial sterilization of milk, effected by subjecting it to a temperature sufficiently high to kill all pathogenic and most of the souring and spore-forming bacteria, but not high enough to produce any physical changes, such as are brought about when the milk is sterilized. A temperature of  $60^{\circ}$  C. continued for fifteen or twenty minutes is usually sufficient, though temperatures as high as  $85^{\circ}$  C. are frequently employed. Enhanced keeping qualities and the destruction of pathogenic organisms are the results of pasteurization.

The chemical substances most frequently employed for sterilization or disinfection<sup>1</sup> are solutions of corrosive sublimate (1 : 1000), carbolic acid (1 : 20), or formalin (1 : 20). A substance that prevents the development of bacteria but does not destroy them is an antiseptic. One that destroys all germs and spores is a germicide or disinfectant. There are many gases, acids, salts, etc., that have antiseptic or germicidal properties.

Bacteria may be removed from a liquid by passing it through a properly constructed filter of un-

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<sup>1</sup> Disinfection is the term applied to sterilization by means of chemicals.

glazed porcelain. The most satisfactory filters in use at present are the Chamberland and the Berkefeld.

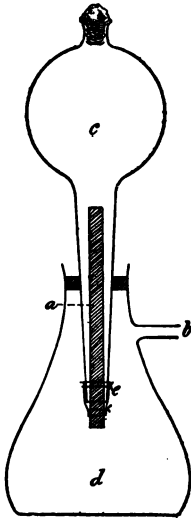


FIG. 20.—Filter: *a*, porcelain bougie; *b*, attachment for suction-pump; *c*, reservoir; *d*, sterile receiver; *e*, rubber tube wired to bougie and reservoir.

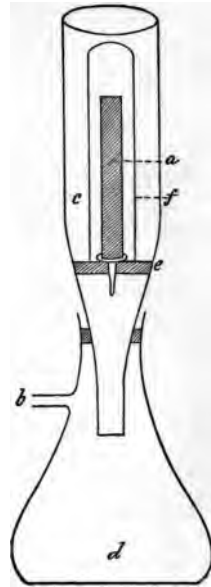


FIG. 21.—Filter: *a*, porcelain bougie; *b*, attachment for suction-pump; *c*, reservoir; *d*, sterile receiver; *e*, perforated rubber collar; *f*, glass chimney for drawing the fluid around the bougie (Page).

Bacteria in the air are unable to pass through a cotton-wool filter, and consequently sterile flasks or

test-tubes stopped with a cotton-wool plug remain sterile indefinitely.

### I. Steam Sterilization.

For objects liable to be injured by dry heat, such as culture-media, fluids, instruments, etc.:

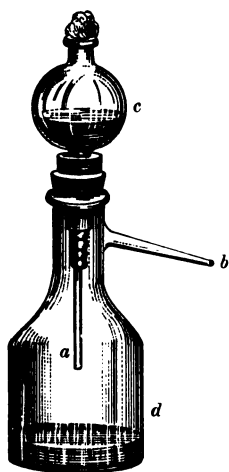


FIG. 22.—Kitasato's filter: *a*, porcelain bougie; *b*, attachment for suction-pump; *c*, reservoir; *d*, sterile receiver.

(*a*) Fill the water-tank of the sterilizer<sup>1</sup> and start the flame.

(*b*) When the chamber is filled with steam place the objects to be sterilized within and close the door.

(*c*) If the objects will not be injured by prolonged heating, allow them to remain for one hour.

(*d*) If prolonged heating is injurious, allow them to remain twenty minutes, and repeat the process three times at intervals of twenty-four hours.

### II. Steam Sterilization under Pressure.

For rapid and effective sterilization. In this method an "autoclave" is used.

(*a*) Adjust the safety-valve at the desired pressure, say thirty pounds.

See that an abundance of water is present, so that the steam will not be superheated.

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<sup>1</sup> An "Arnold" sterilizer is the form usually employed.

(b) Place the objects to be sterilized in the chamber.

(c) Close the door and turn on the steam, first allowing the air present in the chamber to escape.

(d) Bring the temperature to, say  $120^{\circ}$  C. ; allow it to remain there fifteen minutes.

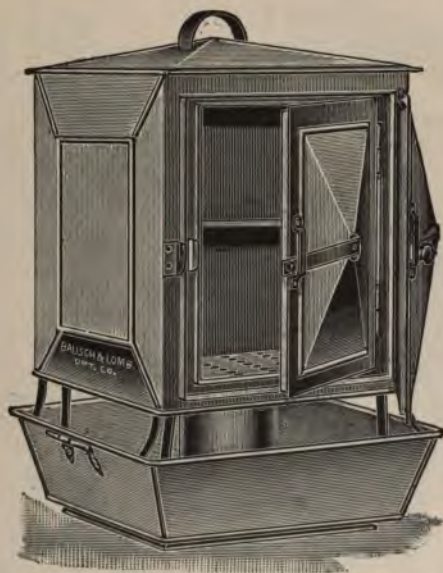


FIG. 23.—Arnold steam sterilizer, Boston Board of Health pattern.

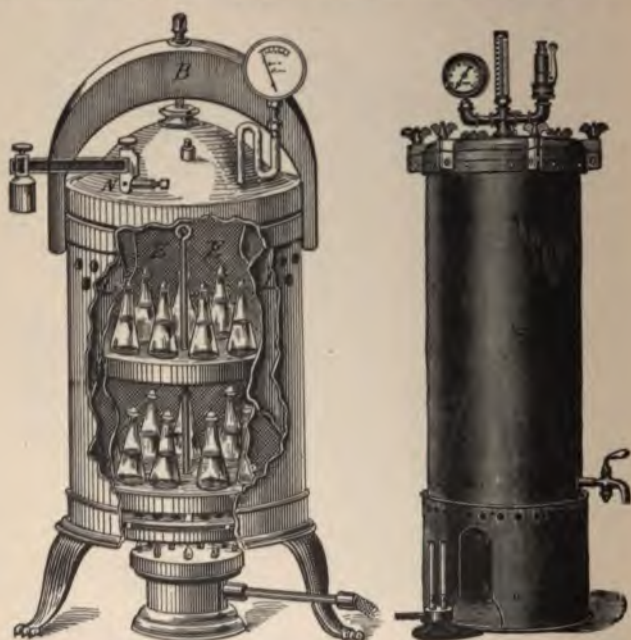
(e) Shut off the steam and allow the apparatus to cool well below  $100^{\circ}$  C. before opening the door or allowing the steam to blow off.

### III. Hot-air Sterilization.

For glassware and other objects not liable to be

injured by dry heat. The oven of an ordinary gas-stove into which a thermometer can be inserted makes an excellent hot-air sterilizer.

(a) Wash thoroughly, using, if necessary, the cleaning mixture recommended on page 14, all



FIGS. 24, 25.—Autoclaves for rapid sterilization by steam under pressure.

flasks, test-tubes, Petri dishes, etc., to be used in the preparation of culture-media.

(b) Plug the flasks and test-tubes with cotton.<sup>1</sup>

<sup>1</sup> "Sliver" obtained from cotton-mills is excellent for plugging test-tubes, etc.

In the case of the test-tubes, the plugs should be tight enough so that the tubes can be lifted by them.

(c) Place in the hot-air sterilizer, close the door, and bring the temperature to  $150^{\circ}$  C.; keep it there

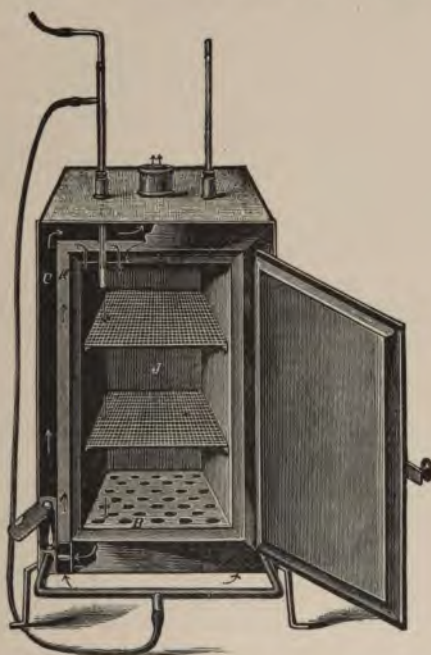


FIG. 26.—Hot-air sterilizer.

for three-quarters of an hour, or until the cotton plugs begin to turn brown.

(d) Allow the oven to cool before opening the door. Place the glassware in a clean place free from dust until used.

## CHAPTER VI.

### PREPARATION OF CULTURE-MEDIA.<sup>1</sup>

I. Bouillon.	II. Gelatin.	III. Agar.
<p>1. Infuse finely chopped lean beef for twenty hours with <i>twice</i> its weight of distilled water in the refrigerator, say 1000 grams of meat, 2000 grams of water.</p> <p>2. Make up weight of meat-infusion (and meat) to original weight by adding water—<i>i. e.</i>, to 3000 grams.</p> <p>3. Filter infusion through cloth to remove meat.</p> <p>4. Weigh infusion, say 1800 grams.</p> <p>5. Set infusion on water-bath, keeping temperature below 60° C.</p> <p>6. Add peptone, 1 per cent., 18 grams; salt, 0.5 per cent., 9 grams.</p> <p>7. After ingredients are dissolved, titrate;<sup>2</sup> reaction probably + 2.3 to + 2.5 per cent.</p> <p>8. Neutralize (Fuller's method).<sup>3</sup></p>	<p>Ditto.</p> <p>Ditto.</p> <p>Ditto.</p> <p>Ditto.</p> <p>Ditto.</p> <p>Ditto, and sheet gelatin, 10 per cent., 180 grams.</p> <p>Ditto, probably + 4.0 to + 5.0 per cent.</p> <p>Ditto.</p>	<p>Boil 30 grams of thread agar in 1 liter of water for half an hour. Make up loss by evaporation to a weight of 1000 grams.</p> <p>1. Infuse finely chopped lean beef for twenty hours with <i>its own</i> weight of distilled water in the refrigerator, say 1000 grams of meat, 1000 grams of water.</p> <p>2. Make up weight of meat-infusion (and meat) to original weight by adding water—<i>i. e.</i>, to 2000 grams.</p> <p>3. Ditto.</p> <p>4. Ditto, say 900 grams.</p> <p>5. Ditto.</p> <p>6. Add peptone, 2 per cent., 18 grams; salt, 1 per cent., 9 grams.</p> <p>7. Ditto, probably + 4.5 to + 4.7 per cent.</p> <p>8. Ditto.</p> <p>To the 900 grams of meat-infusion (containing now peptone and salt) add 900 grams of the 3 per cent. agar-jelly described at the head of this column.</p>

For notes see page 51.

<sup>1</sup> Substantially as recommended by the Bacteriologic Committee of the American Public Health Association. Some minor changes, suggested by Dr. H. W. Hill, in the *Report of the Health Department of Boston for 1898*, have been incorporated without detracting from their value as standard media.

<sup>2</sup> Acid media are denoted by the *plus* sign, and alkaline media by the *minus* sign; the degree of acidity or alkalinity is denoted by parts per hundred. Thus, a medium marked + 1.5 indicates that the medium is acid, and that 1.5 per cent. of  $\frac{n}{1}$  sodium hydroxid is required to make it neutral to phenolphthalein.

<sup>3</sup> Following is Fuller's method of obtaining the degree of reaction of culture-media :

- (a) Prepare a  $\frac{n}{20}$  solution of sodium hydroxid.
- (b) Prepare a  $\frac{n}{20}$  solution of hydrochloric acid.
- (c) Transfer 5 c.c. of the medium to be tested to a porcelain evaporating-dish.
- (d) Add 45 c.c. of distilled water.
- (e) Boil for three minutes.
- (f) Add 1 c.c. of a 0.5 per cent. solution of commercial phenolphthalein in 50 per cent. alcohol.
- (g) Titrate while still hot with the acid or alkali as required, and determine the reaction.

To determine exactly when the neutral point is reached, notice that in bright daylight the first change that can be seen on the addition of alkali is a very faint darkening of the fluid, which on the addition of more alkali becomes a more evident color and develops into what may be described as an Italian pink. A still further addition of alkali suddenly develops a clear and bright pink color, and this is the reaction always to be obtained.

(h) When the reaction has been obtained, calculate the amount necessary to neutralize the bulk of the medium or to produce the required reaction, and add the proper amount of a normal solution of the acid or alkali.



9. Heat over boiling water (or steam) bath thirty minutes.

10. Restore weight lost by evaporation to original weight of filtered meat-infusion, for bouillon and gelatin, and to *twice* that weight for agar—1800 grams in *each case*.

11. Titrate, reaction probably + 0.3 to + 0.5 per cent.

12. Adjust reaction to final point desired, + 1.5 per cent.

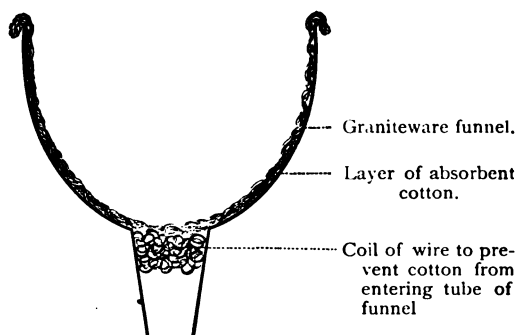


FIG. 27.—Filter for culture-media.

13. Boil five minutes over free flame, with stirring.

14. Add water if necessary to make up loss by evaporation to 1800 grams.

15. Filter through absorbent cotton, passing the filtrate through the filter repeatedly until clear.

16. Titrate to determine whether or not the desired reaction has been maintained.

17. Tube and sterilize for fifteen minutes in the steam sterilizer on three successive days.<sup>1</sup>

Some of the gelatin- and agar-tubes after the

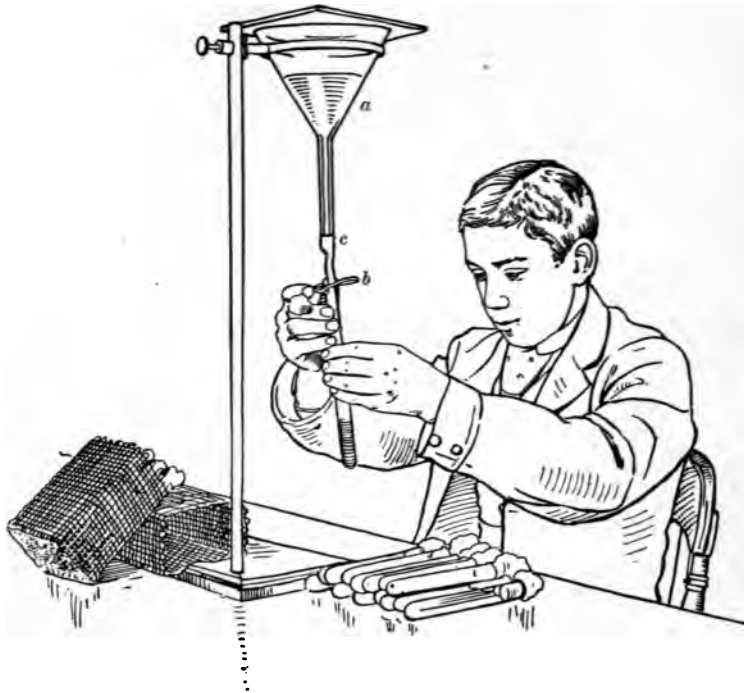


FIG. 28.—Funnel for filling tubes with culture-media : *a*, funnel containing the culture-media in liquid condition ; *b*, pinch-cock by which the flow of fluid into the test-tube is regulated ; *c*, rubber tubing (Warren).

<sup>1</sup> Allow the chamber to fill with steam before placing the media within. Do not leave the media in the sterilizer to cool. Plunge gelatin-tubes into ice-water immediately after each sterilization, in order to maintain a high melting-point.



FIG. 29.—Providence Health Department tube of heavy glass, with etched surface for writing data. These tubes are etched by dipping for thirty seconds in "white acid."



FIG. 30.—Potato-tube (Mallory and Wright).

last sterilization should be allowed to solidify with slanting surfaces.

#### IV. Media from Meat-extracts.

(a) Mix thoroughly the white of 1 egg with 1000 c.c. of water for bouillon or gelatin ; with 500 c.c. of water for agar.

(b) Proceed then as directed in the above table, beginning with No. 4, substituting "white of egg solution" for "infusion." At No. 6 add 0.5 per cent. of Liebig's extract of beef for bouillon or gelatin, 1.0 per cent. for agar.

#### V. Potato.

(a) Select large sound potatoes and wash thoroughly.

(b) Cut off the ends, and with a sterile cork-borer cut out cylinders of the potato a little smaller than the tubes in which they are to be placed. Handle the potatoes under water as much as possible, to prevent darkening of the surface.

(c) Cut the cylinders into two equal parts by a diagonal cut.

(d) Place in cold running water for twelve to eighteen hours. This will usually render the potato neutral.

(If necessary to change the reaction of the potato, steam in a measured quantity of distilled water for one-half hour. Titrate and add the required amount of  $\frac{n}{1}$  sodium hydroxid, and repeat the boiling for thirty minutes.)

(e) Distribute in tubes in the bottom of which a small amount of non-absorbent cotton or a short piece of glass rod has been placed, and sterilize in

the steam sterilizer for thirty minutes on three successive days.

## VI. Dextrose, Lactose, and Saccharose Bouillon.

(a) After filtration of the meat-infusion, prepared as above described, place in an Erlenmeyer flask, and inoculate with a fluid culture of the *Bacillus coli* or an allied gas-producer.

(b) Place in the incubator at  $37.5^{\circ}$  for twenty-four hours.



FIG. 31.—The Smith fermentation-tube.



FIG. 32.—A graduated fermentation-tube.

This removes the meat-sugar.

(c) From this infusion prepare bouillon in the ordinary way.

(d) To the completed broth add 1 per cent. of the required sugar.

(e) Distribute in test-tubes or in fermentation-tubes,<sup>1</sup> and sterilize in the steam sterilizer on three successive days.

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<sup>1</sup> A small test-tube inverted inside a large one will answer for a fermentation-tube.

**VII. Milk and Litmus Milk.**

- (a) Heat fresh milk for fifteen minutes in the steam sterilizer.
- (b) Place in the ice-box over night.
- (c) Siphon off the milk, without cream or sediment.

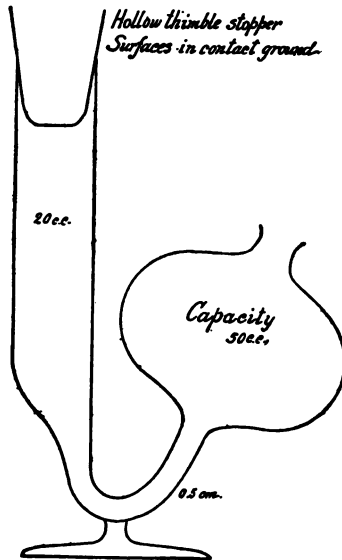


FIG. 33.—The Hill fermentation-tube.

- (d) Titrate.
- (e) If less than 2 per cent. acid to phenolphthalein, place in tubes and sterilize for twenty minutes on four successive days in the steam sterilizer. If more than 2 per cent. acid, adjust to + 1.5 per cent. by the addition of  $\frac{n}{1}$  sodium hydroxid.

(f) A solution of litmus may be added just

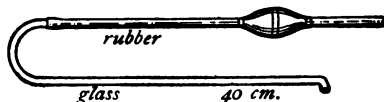


FIG. 34.—Siphon, with one-way valve for starting the flow of serum. The end of the glass tube is turned over to prevent the clot from entering.

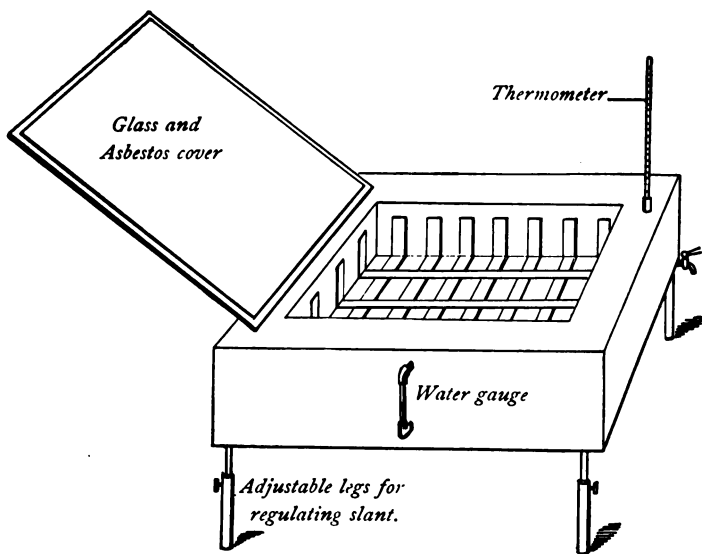


FIG. 35.—Coagulator for blood-serum tubes. Providence Health Department pattern, with wooden rack for holding tubes away from sides. When in use, the four sides and the space below are covered with asbestos boards.

previous to its distribution in tubes, sufficient to give the milk a pale-blue color.

**VIII. Blood-serum** (*Löffler's*).

(a) Receive freshly drawn beef blood in sterile jars.

(b) Allow twenty minutes for coagulation to begin, then with a sterile glass-rod break up any adhesions between the coagulum and the jar.

(c) Allow the jars to stand twenty-four hours in the ice-box.

(d) Siphon off the clear serum.<sup>1</sup>

(e) To 3 parts of serum add 1 part of 1 per cent. dextrose bouillon.<sup>2</sup>

(f) Adjust to + 0.8 per cent.

(g) Distribute in tubes and solidify with a slanting surface by heating for three hours in a blood-serum coagulator.

(h) Sterilize in the steam sterilizer for twenty minutes on three successive days.

---

<sup>1</sup> If the serum is not clear, filter through the coagulum left after the filtration of bouillon, as suggested by Hill.

<sup>2</sup> If 1.25 per cent. glycerin is added also, it seems to prevent the tubes from drying.



## CHAPTER VII.

### CULTURES OF BACTERIA.

OWING to their small size and to their similarity of form, the different species of bacteria cannot be recognized by microscopic examination alone. Recourse must be had to a study of their biologic peculiarities. For this purpose it is necessary to grow them on artificial culture-media. If a single germ is planted on a suitable nutrient medium, and is protected from contamination, it multiplies rapidly and forms a colony. Such a colony is composed of but one species, since all its members are the descendants of a single germ. Cultures made from such a colony are known as "pure cultures," since they contain but one species. From a study of the behavior of these pure cultures, under different conditions, the diagnostic characters of the species are determined.

#### I. BOUILLON CULTURES.

1. Inoculate a tube of bouillon by touching the culture to be studied with a sterile platinum needle, and then dipping the needle in the bouillon.
2. Place at the room-temperature or in the incubator, and examine from day to day, and note any changes as follows :

- (a) Does the bouillon become uniformly clouded, or only at the surface or bottom ?
- (b) Does a pellicle form on the surface or a deposit at the bottom ?



FIG. 36.—Method of holding tubes during inoculation.

- (c) Is the color, odor, or reaction of the bouillon changed ?

## II. GELATIN OR AGAR CULTURES.

### I. "Stab" Cultures.<sup>1</sup>

(1) Sterilize a straight platinum needle and touch it to the culture to be studied ; hold the gelatin- or agar-tube upside down, remove the cotton plug, and stab the needle carefully up through the center of the medium nearly to the bottom of the

---

<sup>1</sup> "Stab" cultures are usually made in gelatin-tubes ; "stroke" cultures, on agar-tubes.

tube; withdraw the needle carefully and replace the cotton plug.

(2) Allow to grow at the room-temperature, if gelatin is used; at either room- or incubator-temperature, if agar is used; examine from day to day.

(3) Note in stab cultures:

(a) Does the growth appear along the line of puncture, at the surface, or in both places?

(b) Is the surface growth abundant or scanty; does it spread over the whole surface or is it confined to the point of puncture; is its margin regular or indented; is it flat or raised, dry or moist; what is its color, luster?

(c) Is the medium changed in color, odor, or condition?

(d) In gelatin cultures, if liquefaction takes place, is it at the surface, deep, or throughout the line of puncture? What is the

form of the liquefied area.

The following terms have been suggested by

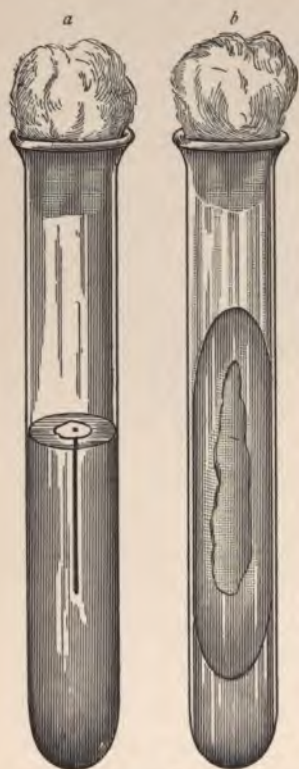


FIG. 37.—*a*, "Stab" culture; *b*, "stroke" culture.

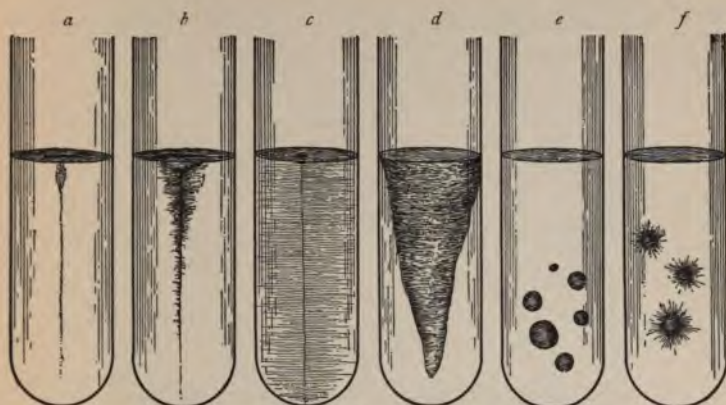


FIG. 38.—Various forms of gelatin "stab" cultures: *a*, *Bacillus typhosus*; *b*, *Bacterium anthracis*; *c*, *Bacillus mycoides*; *d*, *Bacillus mesentericus*; *e*, *Bacillus œdematis*; *f*, *Bacillus radiatus*.

Chester<sup>1</sup> for describing the characters of gelatin stab cultures:

### I. Non-liquefying Cultures.

Line of puncture may be—

- |                      |  |
|----------------------|--|
| <i>Filiform</i> ,    | uniform growth without special characters.                       |
| <i>Nodose</i> ,      | consisting of closely aggregated colonies.                       |
| <i>Beaded</i> ,      | consisting of loosely placed, disjointed colonies.               |
| <i>Papillate</i> ,   | beset with papillate extensions.                                 |
| <i>Echinate</i> ,    | beset with acicular extensions.                                  |
| <i>Villous</i> ,     | beset with short, undivided, hair-like extensions.               |
| <i>Plumose</i> ,     | a delicate feathery growth.                                      |
| <i>Arborescent</i> , | branched or tree-like, beset with branched hair-like extensions. |

<sup>1</sup> *Eleventh Annual Report of the Delaware College Agricultural Experiment Station*, Newark, Delaware, 1898-1899.

## II. Liquefying Cultures.

Line of liquefaction may be—

- |                          |   |
|--------------------------|---|
| <i>Crateriform</i> ,     | a saucer-shaped liquefaction.   |
| <i>Saccate</i> ,         | shape of an elongated sac, tubular,<br>cylindrical.                       |
| <i>Infundibuliform</i> , | shape of a funnel, conical.   |
| <i>Napiform</i> ,        | shape of a turnip.  |
| <i>Fusiform</i> ,        | shape of a parsnip, narrow at either<br>end, broadest below the surface.  |
| <i>Stratiform</i> ,      | liquefaction extending to walls of the<br>tube and downward horizontally. |

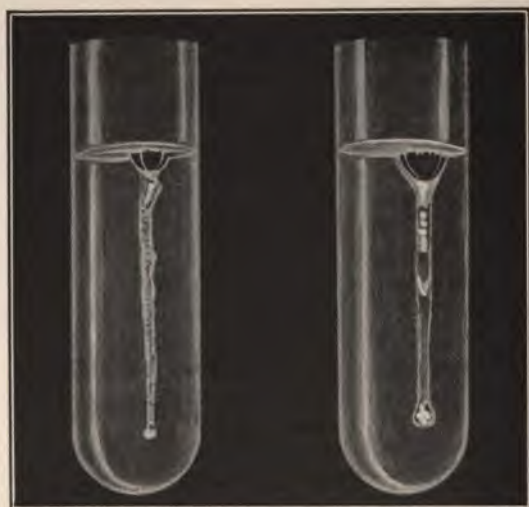


FIG. 39.—*Microspira comma* (Asiatic cholera): gelatin puncture cultures aged forty-eight and sixty hours (Shakespeare).

### 2. "Stroke" Cultures.

(1) Sterilize the platinum needle, touch it to the



FIG. 40.—*Microspira Finkleri*: gelatin puncture cultures aged forty-eight and sixty hours (Shakespeare).



FIG. 41.—*Microspira Metschnikovi*: puncture culture in gelatin forty-eight hours old (Fränkel and Pfeiffer).

culture to be studied, and draw it over the surface of an agar- or gelatin-tube that has been solidified with a slanting surface.

(2) Allow to grow as before, and examine from day to day.

(3) Note :

(a) What are the size and shape of the streak ?

(b) What is the character of the margin ?

(c) Is the growth abundant or scanty, flat or raised, opaque or transparent ?<sup>1</sup>

(d) What is the color, luster ?

(e) Has the color, consistence, or odor of the medium changed ?

### III. POTATO AND BLOOD-SERUM CULTURES.

Stroke cultures are employed in using potato or blood-serum tubes. The methods of study are essentially the same as described for gelatin or agar cultures.

### IV. PLATE CULTURES.

In making plate cultures the nutrient medium is liquefied and a very small portion of the culture to be studied mixed with it ; it is then poured into sterile Petri dishes, solidified, and the colonies allowed to develop. By this method the bacteria are scattered through the medium, and the colonies that develop are the descendants of a single germ. This permits different species to be separated from a mixture in pure culture, and the peculiarities of

---

<sup>1</sup> For descriptive terms, see p. 70, *et seq.*



"colonies" in distinction from "cultures" to be determined.

1. Liquefy three gelatin- or agar-tubes<sup>1</sup> by placing in a water-bath. Gelatin-tubes must not be heated above 40° C. Agar-tubes may be heated to 100° C., but must be cooled to 40° C. before the inoculations are made.

2. With a sterile platinum needle transfer a

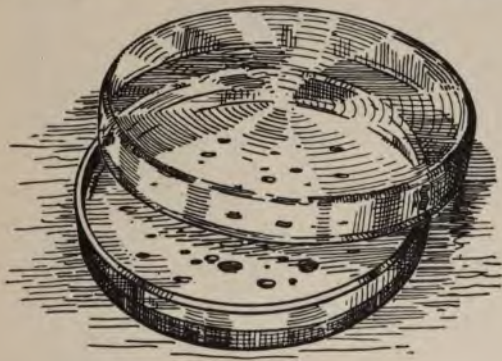


FIG. 42.—Petri dish with colonies.

minute portion of a culture to the liquefied medium in the first tube.

3. Shake the tube thoroughly without producing bubbles in the medium, and transfer a loopful to the second liquefied tube.

4. Shake the second tube, and transfer a loopful to the third tube.

---

<sup>1</sup> Bouillon-tubes or tubes of sterile water may be substituted for the first two gelatin- or agar-tubes when it is desired to make but one plate.





FIG. 43.—Brown University electric water or paraffin bath.<sup>1</sup>

<sup>1</sup> A porcelain-lined dish fitted with a tin cover, provided with four doors. In the figure the cover is raised from the dish to show the thirty candle-power electric lamp which projects through the cover into the water or paraffin below. The lamp is provided with a regulating socket giving

5. Shake the third tube, and pour the contents of the three tubes into sterile Petri dishes, having first passed the lip of each tube through the flame.

6. On the surface of the first Petri dish, before



FIG. 44.—The various appearances of colonies of bacteria under the microscope: *a*, colony of *Bacterium parvum*; *b*, colony of *Bacillus polypiformis*; *c*, colony of *Bacillus radiatus*.

the medium has entirely solidified, press a sterile<sup>1</sup> cover-glass or small piece of mica.

candle-powers from five to thirty. By adjusting the socket to the different candle-powers the requisite temperature of water or paraffin is secured and indicated by the thermometer. When used as a water-bath, the dish is provided with a wire rack to support the tubes to be melted. When used for paraffin, little baskets of wire gauze, containing the specimens to be embedded, are hung about the sides. It is convenient to have the water-bath kept at a temperature of 40° C. In this gelatin-tubes may be melted, or agar-tubes, after being melted at a higher temperature, may be cooled down and kept melted until used.

<sup>1</sup> Sterilize by heating in the flame. Allow it to cool before placing on the plate.

7. Allow to develop as directed for tube cultures, and examine from day to day, or oftener as necessary.

8. In plate cultures note :

(a) Is there a difference in the number of colonies in the three dishes? The use of the three tubes and plates is for the purpose of reducing the number of colonies, so that only a few will be present in the third plate.

(b) Is there more than one kind of colony present?

Notice that colonies on the surface differ from those below the surface, though of the same species. If the culture from which the inoculations were made is a pure culture, and if no germs have been allowed to enter during the process of making the plates, there should be but one kind of colony present.

(c) What are the size, shape, texture, and color of the colonies, both surface and deep? What is the character of their margins?

The following terms are those suggested by Chester for the description of colonies :

### I. Form.

*Punctiform*, dimensions too small for definition.

*Round*, of a more or less circular outline.

*Irregular*.

*Elliptical*.

*Fusiform*, spindle-shaped.

*Cochleate*, spiral or twisted like a shell.

*Ameboid*, very irregular, streaming.

*Mycelioid*, a filamentous colony with the radiate character of a mould.

*Filamentous*, an irregular mass of loosely woven filaments.

*Floccose*, of a dense woolly structure.

*Rhizoid*, of an irregular, branched root-like character.

*Conglomerate*, an aggregate of colonies of similar size and form.

*Toruloid*, an aggregate of colonies like the budding of the yeast plant.

*Rosulate*, like a rosette.

## II. Surface Elevation.

### 1. General character of a surface as a whole.

*Flat*, thin, leafy, spreading over the surface.

*Effused*, spread over the surface as a thin, veily layer more delicate than the preceding.

*Raised*, growth thick with abrupt terraced edges.

*Convex*, surface the segment of a circle, but very flatly convex.

*Pulvinate*, surface the segment of a circle, but decidedly convex.

*Capitate*, surface semi-spherical.

### 2. Detailed characters of surface.

*Smooth*, surface even, without any of the following distinctive characters.

*Alveolate*, honeycombed.

*Punctate*, dotted with punctures.

*Bullate*, blistered.

*Vesicular*, more or less covered with mi-

nute vesicles due to gas formation ;  
more minute than bullate.

*Verrucous*, bearing wart-like prominences.  
*Squamose*, scaly.

*Echinate*, beset with pointed prominences.

*Papillate*, beset with nipple-like processes.

*Rugose*, short irregular folds due to shrinkage.

*Corrugated*, in long folds due to shrinkage.

*Contoured*, a smoothly undulating surface.

*Rimose*, abounding in clefts or cracks.

### III. Microscopic Structure.

1. Refraction weak. Outline and surface of relief not strongly defined.

2. Refraction strong. Outline and surface of relief strongly marked.

(a) Dense, not filamentous colonies.

1. General.

*Amorphous*, without definite structure.

*Hyaline*, clear and colorless.

*Homogeneous*, structure uniform throughout all parts of the colony.

*Homochromous*, colony of uniform color throughout.

2. Granulations or blotchings.

*Finely granular*.

*Coarsely granular*.

*Grumous*, coarser than preceding.

*Moruloid*, segmented.

*Clouded*, having a pale ground with ill-defined patches of a deeper tint.

## 3. Colony marking or striping.

*Reticulate*, in the form of a network.*Areolate*, divided into rather irregular or angular spaces.*Gyrose*, marked by wavy lines.*Marmorated*, traversed by vein-like markings.*Rivulose*, marked by lines like the rivers on a map.*Rimose*, showing cracks or clefts.

## (b) Filamentous colonies.

*Filamentous*, as already defined.*Floccose*, composed of filaments densely packed.*Curled*, filaments in parallel strands.

## IV. Edges of Colonies.

*Entire*, without toothing or division.*Undulate*, wavy.*Repand*, like the border of an open umbrella.*Erose*, as if gnawed, irregularly toothed.*Lobate*, divided into lobes.*Lobulate*, minutely lobate.*Auriculate*, with ear-like lobes.*Lacerate*, irregularly cleft, as if torn.*Fimbriate*, fringed.*Ciliate*, hair-like extensions.

<i>Tufted,</i>	} as already defined.
<i>Filamentous,</i>	
<i>Floccose,</i>	
<i>Curled,</i>	

**V Optical Characters.**

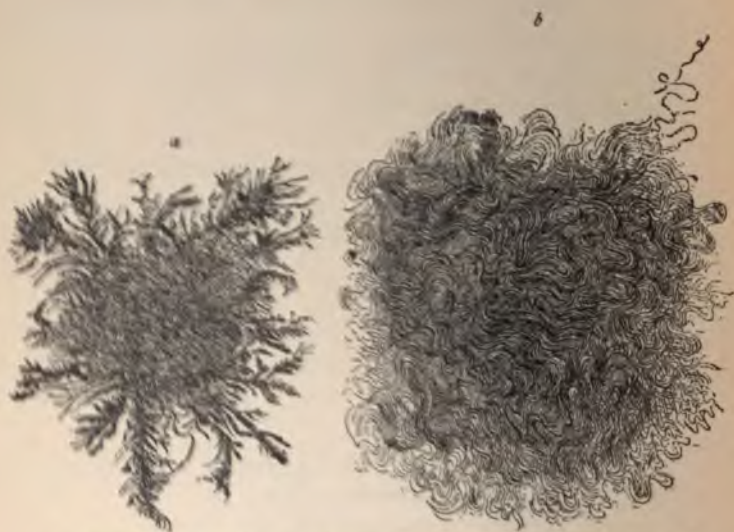
*Transparent.*

*Vitreous*, transparent and colorless.

*Oleaginous*, transparent and yellow.

*Resinous*, transparent and brown.

*Translucent.*



FIGS. 45, 46.—The various appearances of colonies of bacteria under the microscope: *a*, colony of *Bacillus muscoides* (Liborius); *b*, colony of *Bacterium anthracis* (Flügge).

*Porcelaneous*, translucent and white.

*Opalescent*, translucent, grayish white by reflected light, smoky brown by transmitted light.

*Nacreous*, translucent, grayish white with pearly luster.

*Sebaceous*, translucent, yellowish or grayish white.

*Butyrous*, translucent and yellow.

*Ceraceous*, translucent and wax-colored.

*Opaque*.

*Cretaceous*, opaque and white ; chalky.

*Dull*, without luster.

*Glistening*.

*Fluorescent*.

*Iridescent*.

*Phosphorescent*.

(*d*) Is there growth beneath the cover-glass or mica plate? This determines, roughly, whether they require oxygen for their growth or not (aërobic or anaërobic).

(*e*) In the gelatin plates is the gelatin liquefied, and what is the nature of the liquefaction?

## V. IMPRESSION OR ADHESIVE PREPARATIONS OF COLONIES.

An entire colony of bacteria may be preserved as a microscopic specimen.

(*a*) Slightly warm a clean cover-glass.

(*b*) Lay it upon the surface of the gelatin or agar containing the colonies. Apply sufficient pressure to remove all air-bubbles, but not enough to disturb the colony.

(*c*) Remove the cover, gently lifting it from one side. The colonies will adhere to the glass.



(d) Dry, fix, stain, and mount as for ordinary preparations.

Museum preparations of gelatin or agar tube or plate cultures may be made by exposing the cultures to formaldehyd vapor until the growth is killed, and then sealing the tubes or plates tightly with sealing-wax or paraffin.



FIG. 47.—*Bacterium tuberculosis*: adhesive preparation from a fourteen-day blood-serum culture;  $\times 100$  (Fränkel and Pfeiffer).

#### VI. CULTURES IN THE FERMENTATION-TUBE.

This method is for the purpose of studying gas formation, and for the study of the aërobic or anaërobic properties of organisms.

(a) Prepare fermentation-tubes with dextrose, lactose, or saccharose bouillon.

(b) Inoculate the tubes by floating a little of the

culture to be studied on the fluid in the bulb with a sterile needle.

(c) In the case of gas formation, at the end of every twenty-four hours, for several days, mark the level of the fluid in the closed branch upon the tube or measure it with a millimeter scale.

(d) Record the result in percentages of the length



FIG. 48.—*Bacterium anthracis*: colony three days old upon a gelatin plate; adhesive preparation;  $\times 1000$  (Fränkel and Pfeiffer).

of the closed branch. If 1 cm. of gas forms in a 10 cm. tube, 10 per cent. of gas is said to have formed.

(e) To test the relative amount of carbon dioxid and hydrogen present. Fill the bulb completely with a 2 per cent. solution of sodium hydroxid. Place the thumb over the mouth of the bulb, and

run the mixture six or eight times through the length of the tube, returning the remaining gas to the closed branch before removing the thumb. Measure the amount of gas remaining ; the difference between this and the former measurement shows in millimeters the amount of carbon dioxid absorbed by the alkali. The remaining gas, mostly hydrogen, may be transferred to the bulb and exploded by a flame. The proportion of hydrogen to carbon dioxid is usually expressed in the form of a fraction called the gas formula,  $\frac{H}{CO_2}$ .

The fermentation-tube affords a ready method of determining the oxygen requirement of bacteria. Growth, indicated by cloudiness, in the bulb only, is to be found only among obligatory aërobes ; in the closed branch only, among obligatory anaërobes ; while growth in both, only among the facultative anaërobes.

## VII. ANAËROBIC CULTURES.

Growth under the mica plate or cover-glass<sup>1</sup> and in the fermentation-tube are methods for the determination of the aërobic properties of organisms. For the growth of strictly anaërobic forms special methods have been devised :

**1.** Place the cultures in a vessel from which the air can be withdrawn and hydrogen substituted.

**2. Buchner's Method.**—Use two test-tubes, one inside the other. The outer one is partially filled

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<sup>1</sup> See page 75.

with pyrogallic acid made alkaline with sodium hydrate, and is sealed tightly with a rubber stopper. The inner tube contains the culture. The oxygen is absorbed by the mixture in the outer tube.

**3. Wright's Method.**—Make an ordinary culture in a test-tube. Clip off any superfluous cotton from the plug, and push the plug into the tube so



FIG. 49.—Novy's jars for anaerobic cultures.

that it lies 1 centimeter below the mouth. For test-tubes  $6 \times \frac{3}{4}$  inches, run into the cotton plug, from a pipet, approximately  $\frac{1}{2}$  c.c. of a freshly prepared solution of pyrogallic acid (1 part of acid, 1 part of water), and then approximately 1 c.c. of a solution of sodium hydrate (1 part of sodium hydrate, 2 parts of water). Quickly insert a rubber stopper into the tube.

**4.** Make a hanging-drop culture. On one side of the cover-glass introduce a little pyrogallic acid, and on the other side a little sodium hydrate, so that it runs around and unites with the acid. Seal with vaselin.

5. Distribute the germs to be studied in bouillon or in liquefied gelatin or agar, and draw some of the solution into sterile pieces of glass tubing of small caliber. When the tube is full seal the ends in a flame.

6. Put large quantities of culture-medium in the tubes and puncture deeply. The surface of the medium is then covered with sterile oil.

7. **Park's Method.**—Cover the culture-medium with melted paraffin.<sup>1</sup> Sterilize by the ordinary methods, and when cool enough for inoculation, but before the paraffin solidifies, inoculate through the paraffin into the medium below.

Wright recommends<sup>2</sup> the following precautions in growing anaërobic bacteria :

1. The medium should contain 1 per cent. of glucose, and should be boiled and cooled immediately before inoculation.

2. The medium should be freshly prepared.

3. The reaction should not be more acid to phenolphthalein than +1.5. With 1 per cent. glucose bouillon, growth is better if the reaction is nearer the neutral point of phenolphthalein than +1.5.

#### VIII. DEMONSTRATION OF LIQUEFYING FERMENT.

(a) Inoculate several gelatin-tubes with *Bacillus prodigiosus*.

(b) Allow them to grow until all the gelatin is liquefied.

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<sup>1</sup> Melting point, 42° C.

<sup>2</sup> *Jour. Boston. Soc. Med. Sci.*, vol. v., No. 4, p. 114.

(c) Add  $\frac{1}{2}$  c.c. of chloroform, or 5 per cent. carbolic acid, to each tube, shake thoroughly, and filter.

(d) Add the filtrate, now containing no living bacteria, to tubes of sterile gelatin. Note the liquefaction that takes place, caused by the ferment produced by the bacteria in the first set of tubes.

#### IX. ISOLATION OF SPECIES.

Given a bouillon culture, containing three species of bacteria, to isolate the species.<sup>1</sup>

(a) Liquefy three gelatin or agar tubes and number 1, 2, and 3 respectively.<sup>2</sup>

(b) Transfer a minute loopful of the bouillon culture to tube No. 1.

(c) Shake thoroughly, and transfer 2 loopfuls from tube No. 1 to tube No. 2.

(a) Shake and transfer 3 loopfuls from tube No. 2 to tube No. 3.

(e) Flame the lips of the tubes and pour their contents into sterile Petri dishes.

(f) Examine in twelve to twenty-four hours.

(g) Select the dish in which the colonies are well developed, and in which they have not run together; look for three kinds of colonies.

If more than three kinds are present, it shows

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<sup>1</sup> This method is applicable to the separation of species from any fluid.

<sup>2</sup> Tubes of bouillon or sterile water may be substituted for tubes Nos. 1 and 2, in which case, of course, but one plate can be made—*i. e.*, from tube No. 3.

that others have been allowed to enter through carelessness in manipulation.

(h) Record the appearance of the different colonies, and inoculate them as pure cultures in tubes of culture-media. Study them as directed in the following chapter.

## CHAPTER VIII.

### DETERMINATION OF SPECIES.

#### I. MORPHOLOGY AND LIFE-HISTORY OF A SPECIES.

THE following points in the morphology and life-history of any form of bacterium must be known before it can be fully described or assigned a place in any particular species.<sup>1</sup>

##### I. SOURCE AND HABITAT.

##### II. MORPHOLOGIC CHARACTERS.

1. Form.
2. Dimensions.
3. Manner of grouping and arrangement in the growths.
4. Staining powers : (a) with aqueous stains; (b) by Gram's method.
5. Presence or absence of capsule.
6. Presence or absence of flagella (motility).
7. Spore-formation.
8. Tendency to pleomorphism.
9. Involution and degeneration forms.

##### III. BIOLOGIC CHARACTERS.

*A. Cultural characteristics*, mode of growth in and upon—

1. Bouillon.
2. Gelatin plates (single colonies, surface and deep).
3. Gelatin-tubes.
4. Agar plates (single colonies, surface and deep).

---

<sup>1</sup> These are the points recommended by the Committee of Bacteriologists of the American Public Health Association.



5. Agar-tubes.
6. Potato.
7. Milk.
8. Blood-serum.

*B. Biochemic features.*

1. Temperature relationship (activity of growth at 18°–22° C. and at 36°–38° C. and thermal death-point).
2. Relation to free oxygen (aërobic and anaërobic growth).
3. Relation of growth to acidity and alkalinity of media.
4. Action upon gelatin (presence or absence of liquefaction).
5. Action upon proteids (milk and serum).
6. Action upon carbohydrates (fermentation and gas formation).
7. Action upon nitrates.
8. Production of indol.
9. Production of acid or alkali.
10. Pigment formation.
11. Development of odor.

*C. Pathogenesis.*

The following tests are of value in certain cases :

I. MORPHOLOGIC.

1. Staining reactions with special stains.
2. Study of flagella by special stains.
3. Permanency of morphologic characters after long-continued growth and successive transplantation upon artificial media.
4. Photographic reproductions of isolated bacteria.
5. Cover-glass impressions.

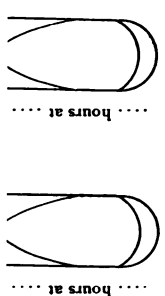
II. PHYSIOLOGIC.

*A. Cultural characteristics, mode of growth in or upon—*

1. Litmus gelatin.
2. Löffler's blood-serum.
3. Synthesized media.
4. Photographic reproduction of characteristic cultures.





MEDIUM. Surface relief. Light transmission. Color. Luster. Consistency. <i>Change in medium.</i> Color. Consistency. Odor.		Gas production.		 hours at ..... hours at .....	
NITRATE BROTH. Reduction of nitrates. Dextrose-free bouillon. Indol production.					
MILK. <i>Curd, curdles or does not curdle in ..... days at 18°-20° C. or 36°-38° C.</i> <i>“ “ “ “ on boiling after ..... days at .....° C.</i> <i>hard or soft, in one mass or in fragments, gas-bubbles</i> <i>changed or unchanged by boiling.</i> <i>Whey separates from curd or not, amount, transparent or turbid.</i> <i>Reaction, ..... in ..... days at .....° C.</i> <i>Color, ..... becomes gradually transparent without forming curds.</i> <i>Digestion complete in ..... days at .....° C.</i> <i>effects of boiling, odor, clear or cloudy, watery or viscid.</i> <i>reaction at end of digestion .....</i>					
SUGAR BOUILLON IN FERMENTATION-TUBES		Amount of gas in % formed at 18°-20° C.		Reaction of fluid in .....° C. after ..... days at .....° C.	
Dextrose ..... Lactose ..... Saccharose .....		in 1 d., 2 ds., 3 ds., ..... days in 1 d., 2 ds., 3 ds., ..... days		CO <sub>2</sub> % ..... H % .....	
PIGMENT, developed in presence or absence of oxygen in ..... cultures at .....° C. in ..... hours. color, ..... changed to ..... by acid or alkali. soluble in ..... spectrum .....		PATHOGENESIS.		Pelli- cle. .... Opac- ity. .... Color. ....	
OPTIMUM TEMPERATURE .....° C. GROWTH LIMITS .....° C. to .....° C. THERMAL DEATH-POINT .....° C., time of exposure ..... minutes.		RELATION OF GROWTH TO ACIDITY OR ALKALINITY OF MEDIUM. .... % acid to .... % alkaline.			
PRODUCTION OF ACIDS OR ALKALIES. Carbohydrates absent or present.		RELATION TO FREE OXYGEN. <i>Obligatory anaërobe.</i> <i>Facultative anaërobe.</i> <i>Obligatory anaërobe.</i>			

in the hanging-drop, preferably in a fresh medium inoculated with a very small amount of the culture to be examined. It is desirable that the form and grouping be determined in bouillon, gelatin, and on agar, and that any variation found upon the examination of the growth on other media be accurately noted.

(b) **Test for Motility.**—For the study of motility the hanging-drop preparations should be made from young cultures grown at or near the optimum temperature for only a few (six to eighteen) hours.

(c) **Tests for Spores.**—The tests for the presence of spores are :

(a) Do colonies develop from cultures which have been subjected to a temperature of 80° C. for ten minutes?

(b) Are there highly refracting bodies within the bacteria in unstained preparations, and can they be demonstrated by the spore-staining methods?

Cultures to be tested should be grown for forty-eight hours in bouillon, and when possible at 36°–38° C. Three loopfuls of this culture after agitation are transferred to tubes containing 10 c.c. of bouillon. This is exposed to a temperature of 80° C. for ten minutes, and then placed under conditions favorable for the development of any of the organisms which may have survived.

(d) **Pleomorphism.**—In regard to pleomorphism, attention is called to the variations in size and shape brought about by the following conditions of growth :

(a) At different temperatures.

- (b) Upon or in media of different composition.
- (c) Upon or in media of different degrees of acidity and alkalinity.
- (d) In cultures of different ages.
- (e) As well as to the variations in the size and shape of different individual bacteria obtained from one culture and appearing often in the same field of view—*i. e.*, subjected to exactly the same conditions of growth.

**(e) Determination of the Thermal Death-point.**—In determining the thermal death-point, the facts required to be known are (1) the time of exposure to heat, (2) the presence or absence of moisture, (3) the presence or absence of spores, (4) the age of the culture, (5) the amount of the culture used for the tests, and (6) the character of the containing vessel.

The temperature required to destroy the species under consideration is to be determined within 2 degrees C.; thus, if samples are exposed to temperatures of 50°, 52°, 54°, 56°, 58°, and 60° C., and it is found that development in a suitable medium occurs after exposure to 56° C., but not after exposure to 58° and 60° C., the thermal death-point is to be given as 58° C., although further study might show it to be somewhat less than this.

**(f) Relation to Free Oxygen.**—For the methods of determining the aërobic properties of organisms, see under Anaërobic Cultures, on page 78.

**(g) Relation to Acidity and Alkalinity of Media.**—In determining the relation of growth to

acidity and alkalinity of media, all that is necessary is to add to tubes containing equal quantities of any of the usual media a calculated amount of a standardized solution of hydrochloric acid or of sodium hydroxid to obtain the desired reaction. A record should be made of cultures upon at least one medium reacting + 3 per cent., + 1.5 per cent., neutral, and - 1.5 per cent. to phenolphthalein.

(h) **Action upon Carbohydrates.**—For the methods of determining the action upon carbohydrates and for measuring the gas production, see under Cultures in the Fermentation-tube, page 76.

(i) **Action upon Nitrates.**—To determine the power of certain bacteria to reduce nitrates to nitrites and ammonia, incubate them for seven days at 20° C. in the following :

#### NITRATE BROTH.

Peptone,	1 gm. ;
Potassium nitrate,	0.2 gm. ;
Tap water, <sup>1</sup>	1000 c. c.

Submit the inoculated tubes and also several uninoculated control-tubes to the following tests for nitrites :

Prepare two solutions :

I. Naphthylamin,	0.1 gm. ;
Distilled water,	20 c. c.

Boil until the naphthylamin is dissolved ; cool,

---

<sup>1</sup> Better than distilled water because of the other salts present, which favor the growth of the bacteria.

filter, and add the filtrate to 150 c.c. of dilute (1 to 16) hydric acetate.

- II. Sulphanilic acid, 0.5 c.c.;  
Dilute (1 to 16) hydric acetate, 150 c.c.

Keep these solutions in separate glass bottles, tightly stoppered, and mix in equal parts before use.

To 3 c.c. of the solution to be tested, in a perfectly clean test-tube, add gradually 2 c.c. of the test-solution. A red color develops of an intensity in proportion to the amount of nitrites present. The appearance of the color may be hastened by heating.

If this test shows the presence of nitrites, test one-half of the remaining solution for ammonia with Nessler's solution.<sup>1</sup>

The presence of ammonia is shown by the immediate development of a yellow color or precipitate on the addition of a few drops of the test-solution.

When these tests are positive our inquiry has been answered. When negative the nitrates may have remained unchanged or may have been reduced to free nitrogen. It is therefore necessary to determine whether the nitrates are still present or not, as follows :

(a) Invert the tube containing the culture and evaporate to dryness the small amount of culture remaining on the inside of the tube.

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<sup>1</sup> See Appendix, page 174.



(*b*) Add, in order, phenol-sulphonic acid,<sup>1</sup> water, and sodium hydrate. A yellow color shows the presence of nitrates.

(*j*) **Acid or Alkali Production.**—For the determination of acid or alkali production, cultures are made on solid media to which have been added 1 per cent. of dextrose, saccharose, or lactose, and a sufficient quantity of litmus solution to produce a blue tinge. A pink coloration of the colony or a reddening of the surrounding medium indicates acid production.

(*k*) **Test for Indol Production.**—For the determination of indol production, incubate the species to be tested in the dextrose-free bouillon described on page 56. Add to the tube of culture 1 c.c. of a 0.01–0.02 per cent. solution of sodium or potassium nitrite. To each 10 c.c. of medium add one drop of concentrated sulphuric acid. A red coloration indicates indol. If no result is obtained at once, it is well to allow the tube to stand for one hour.

**Pathogenesis.**—In Chapter VIII. are described the various methods of determining the pathogenic properties of bacteria.

1. When a given form grows only at or below 18° to 20° C. inoculation should be made into the dorsal lymph-sac of a frog, using about 1 per cent. of the body-weight of a bouillon culture seven days old.

2. When a given species grows at 35° C. or upward, inoculation should be made into the peri-

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<sup>1</sup> See Appendix, page 174.

toneal cavity of a white or ordinary house mouse, using 1 per cent. of the body-weight of a forty-eight hour bouillon culture. If the mouse is killed, it is well to try the culture on guinea-pigs or rabbits. A careful autopsy should be made in all cases, cultures taken from the organs, and all pathogenic changes noted.

## II. DETERMINATION OF THE NAME OF A SPECIES.

When the points mentioned at the beginning of this chapter have been determined, the name of the species, if it has been described and named, can be found by referring to the bacterial analysis tables in Migula's *System der Bakterien*, Sternberg's *Manual of Bacteriology*, Flüggé's *Die Mikroorganismen*, or Chester's "Studies in Systematic Bacteriology" in the ninth, tenth, and eleventh *Reports of the Delaware College Agricultural Experiment Station*, 1897, 1898, and 1899.

The following table, adapted from Migula, may assist in the determination of the name of a species :

### BACTERIA.

#### I. Family. Coccaceæ.

##### 1. Genus. Streptococcus.

##### I. Grow on gelatin.

##### 1. Colonies white.

##### A. Do not liquefy gelatin.

##### (a) No growth on the surface in gelatin stab cultures.

S. pyogenes.

S. equi.

S. cystitidis.

*S. urinæ.*

*S. acidi lactici.*

(b) Growth on the surface and along the  
puncture in gelatin stab cultures.

*S. mastitidis.*

*S. mirabilis.*

B. Liquefy gelatin.

*S. septicus.*

*S. morbi brightii.*

*S. gracilis.*

*S. vermiformis.*

*S. albus.*

2. Colonies colored.

*S. cerasinus.*

*S. citreus.*

II. Do not grow on gelatin.

*S. giganteus.*

2. Genus. *Micrococcus.*

I. Grow on gelatin.

1. White.

A. Do not liquefy gelatin.

*M. candicans.*

*M. tardissimus.*

*M. tardus.*

*M. plumosus.*

*M. viticulosus.*

*M. stellatus.*

*M. tenuissimus.*

*M. albocretus (cereus albus).*

*M. ureæ.*

*M. coryzæ.*

*M. salivarius.*

- M. lacticus.
- M. acidi lactici.
- M. phosphoreus.
- M. catarrhalis.
- M. bovis.
- M. similis.
- B. Liquefy gelatin.
  - M. pyogenes.
  - M. ovis.
  - M. foetidus.
  - M. conoideus.
  - M. liquefaciens.
  - M. radiatus.
  - M. faviformis.
  - M. amplus.
  - M. dissimilis.
- 2. Yellow.
  - A. Do not liquefy gelatin.
    - M. aurantiacus.
    - M. tardigradus.
    - M. luteus.
    - M. cereus.
    - M. versicolor.
    - M. varians.
  - B. Liquefy gelatin.
    - M. aureus (S. pyogenes aureus).
    - M. beri-beri.
    - M. fuscus.
    - M. coronatus.
    - M. conjunctivitis.
    - M. tardus.
    - M. flavus.

*M. desidens.*  
*M. conglomeratus.*  
*M. citreus* (*S. pyogenes citreus*).  
*M. citrinus.*  
*M. corrugatus.*  
*M. mollis.*

## 3. Red.

A. Do not liquefy gelatin.

*M. cinnabareus.*  
*M. carneus.*

B. Liquefy gelatin.

*M. roseus.*  
*M. rosaceus.*  
*M. carnicolor.*  
*M. fragilis.*

## 4. Blue and violet.

*M. violaceus.*  
*M. cyaneus.*

## II. Do not grow on gelatin.

*M. gonorrhœæ.*  
*M. intracellularis.*  
*M. subflavidus.*  
*M. rugatus.*  
*M. cuniculorum.*  
*M. progrediens.*  
*M. tenuis.*  
*M. nitrosus.*  
*M. fœtidus.*

3. Genus. *Sarcina*.

I. Grow on gelatin.

1. White.

*Sarcina alba.*

- 2. Yellow.
    - A. Do not liquefy gelatin.
      - Sarcina lutea*.
      - Sarcina ventriculi*.
    - B. Liquefy gelatin.
      - Sarcina liquefaciens*.
      - Sarcina aurantiaca*.
- II. Family. *Bacteriaceæ*.
  - I. Genus. *Bacterium*.
    - I. Form spores.
      - 1. Spores polar.
        - Bact. anthracis*.
        - Bact. anthracoides*.
        - Bact. subtile*.
      - 2. Spores central.
        - Bact. carotarum*.
        - Bact. angulans*.
    - 3. Position of spores undetermined.
      - A. Grow on gelatin at the room-temperature.
        - (a) Colonies white.
          - 1. Do not liquefy gelatin.
            - Bact. acidi lactici*.
            - Bact. coprogenes*.
          - 2. Liquefy gelatin.
            - (1) Center of colonies plainly floccose.
              - Bact. vermiculare*.
            - (2) Colonies not floccose.
              - Bact. tricomii*.
              - Bact. nephritidis*.
              - Bact. sempervivum*.
              - Bact. lacteum*.

(b) Colonies colored.

Bact. brunneum.

B. Do not grow on gelatin at the room-temperature.

Bact. termophilum.

II. Spore formation not observed.

I. Grow well on gelatin.

A. Form no pigment.

(a) Do not liquefy gelatin.

1. Stained by Gram's method.

Bact. pneumoniae.

Bact. proteus.

Bact. rhusiopathiae.

Bact. murisepticum.

Bact. lacticum.

Bact. parvum.

2. Do not stain by Gram's method.

Bact. pneumonicum.

Bact. rhinoscleromatis.

Bact. faschingii.

Bact. endocarditidis.

Bact. cuniculicidi.

Bact. suicida.

Bact. palumbarium.

Bact. ribberti.

Bact. cuniculi.

Bact. pseudotuberculosis.

Bact. columbarum.

Bact. aërogenes.

Bact. aceti.

Bact. salivæ.

3. Gram's stain undetermined.

Bact. capsulatum.

Bact. keratomalaciæ.  
Bact. felis.  
Bact. pyogenes.  
Bact. welchii (aërogenes capsu-  
latus).  
Bact. bienstockii.  
Bact. laerii.  
Bact. ubiquitum.  
Bact. candicans.  
Bact. phosphorescens.  
Bact. giardi.

(b) Liquefy gelatin.

Bact. bovis.  
Bact. vaginali.  
Bact. varicosum.  
Bact. buccale.

B. Colonies yellow.

(a) Do not liquefy gelatin.

Bact. erythrogenes.  
Bact. citreum.  
Bact. aurescens.

(b) Liquefy gelatin.

Bact. arborescens.  
Bact. aquatile.  
Bact. chlorinum.  
Bact. aureum.

C. Colonies red.

Bact. mycoides.  
Bact. pyocinnabareum.

D. Colonies blue or violet.

Bact. cœruleum.  
Bact. amethystinum.



2. Do not grow well on gelatin at room-temperature.

Bact. tuberculosis.

Bact. tuberculosis avium.

Bact. lepræ.

Bact. syphilidis.

Bact. smegmatis.

Bact. mallei.

Bact. diphtheriæ.

Bact. xerosis.

Bact. pseudodiphtheriticum.

Bact. vaginæ.

Bact. influenzæ.

Bact. pseudoinfluenzæ.

2. Genus. Bacillus.

- I. Form spores.

1. Spores polar.

B. ramosus.

2. Spores central or oblique.

B. subtilis.

B. megaterium.

3. Position of spores undetermined.

- A. White or dirty white on gelatin.

- (a) Do not liquefy gelatin.

B. spermophilinus.

B. polypiformis.

B. muscoides.

- (b) Liquefy gelatin.

1. Stain by Gram's method.

B. mycoides.

B. gracilis.

B. granulosus.

- [illegible]

- B. coli.*
- B. pestis.*
- B. avium.*
- B. suispestifer.*
- B. zee.*
- B. glacialis.*
- 2. Gram's stain undetermined.
  - B. aurum.*
  - B. solanacearum.*
  - B. vaseonii.*
  - B. arvicolorum.*
  - B. sorghi.*
  - B. copii.*
- 3. Does not gelatin.
  - Stain by Gram's method.
    - B. asenteriae.*
- 4. Do not stain by Gram's method.
  - B. pseudotuberculosis.*
  - B. maceae.*
  - B. agilis.*
  - B. caltropidus.*
- 5. Gram's stain undetermined.
  - B. anthracis.*
  - B. sulfureus.*
  - B. liquidus.*
  - B. diffusus.*
  - B. nubilis.*
  - B. reticularis.*
  - B. diaphanus.*
  - B. mirabilis.*
  - B. gasoformans.*
  - B. delicatulus.*

- B. cloacæ.
- B. hyalinus.
- B. superficialis.
- B. Colonies yellow.
  - B. arborescens.
- C. Colonies red.
  - B. prodigiosus.
  - B. indicus.
  - B. plymouthensis.
  - B. rubescens.
- 2. Grow on gelatin. Phosphorescent.
  - B. phosphorescens.
  - B. fischeri.
  - B. phosphoricus.
- 3. Do not grow on gelatin.
  - B. equi.
- 3. Genus. *Pseudomonas*.
  - I. Grow on gelatin.
    - 1. Colonies white. Form no pigment.
      - P. litoralis.
    - 2. Form fluorescent pigment.
      - A. Do not liquefy gelatin.
        - P. alba.
        - P. tenuis.
        - P. eisenbergii.
        - P. stewarti.
      - B. Liquefy gelatin.
        - P. æruginosa (B. pyocyaneus).
        - P. fluorescens.
        - P. minutissima.
    - 3. Colonies blue or violet.
      - P. ianthina.

**P. pseudianthina.**

**P. laurentia.**

#### 4. Phosphorescent species.

**P. javanica.**

II. Do not grow on gelatin.

*P. europæa.*

**P. javaniensis.**

### III. Family Spirillaceæ.

1. Genus. Spirosonia.

*Spirosoma nasale.*

**Spirosoma linguale.**

*Spirosoma aureum.*

**Spirosoma flavum.**

**Spirosoma flavescens.**

**Spirosoma attenuatum.**

*Spirosoma gregarium.*

2. Genus. *Microspira*.

I. Not phosphorescent.

1. Do not liquefy gelatin.

**Microspira canalis.**

**Microspira saprophiles.**

**Microspira tonsillaris.**

2. Liquefy gelatin.

**Microspira comma.**

**Microspira metschnikovi.**

**Microspira finkleri.**

**Microspira sputigena.**

*Microspira marina.*

## II. Phosphorescent.

**Microspira dunbari.**

**Microspira coronata.**

**Microspira annularis.**

*Microspira glutinosa.*  
*Microspira delgadensis.*  
*Microspira tuberosa.*  
*Microspira degenerans.*  
*Microspira luminosa.*  
*Microspira caraibica.*  
*Microspira papillaris.*

### III. CLASSIFICATION OF BACTERIA BY GROUPS.

Systematic bacteriology is at present in a state of chaos. Many times the most that can be done in attempting to classify a new species, is to refer it to some group, the members of which have certain characters in common, and are probably the descendants of one ancestral type. Chester has proposed<sup>1</sup> a synopsis of several groups of bacteria which, slightly modified, is given below.

#### BACTERIUM.

##### I. Spore-formers.

1. No growth at room-temperature or below 22°-25° C.

##### THERMOPHILIC GROUP.

Bact. termophilum type.

2. Grow at room-temperature.

A. Do not liquefy gelatin.

##### BACT. FÆCALIS GROUP.

Bact. subtile type.

B. Liquefy gelatin.

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<sup>1</sup> *Eleventh Annual Report of the Delaware College Agricultural Experiment Station for 1898-99.*

## ANTHRAX GROUP.

Bact. anthracis type.

II. Spore formation not observed. Aërobic and facultative anaërobic.

1. Do not liquefy gelatin.

A. Do not stain by Gram's method.

(a) Obligate aërobic.

## ACETIC FERMENT GROUP.

Bact. aceti type.

(b) Aërobic and facultative anaërobic.

1. Gas generated in glucose bouillon.

a. Gas generated in lactose bouillon.

## BACT. AËROGENES GROUP.

Bact. aërogenes type.

b. Little or no gas generated in lactose bouillon.

## FRIEDLANDER GROUP.

Bact. pneumonicum type.

2. No gas generated in glucose bouillon.

a. Coagulate milk.

## FOWL-CHOLERA GROUP.

Bact. cuniculcida type.

b. Do not coagulate milk.

## SWINE-PLAGUE GROUP.

Bact. suicida type.

B. Stain by Gram's method.

Gas generated in glucose bouillon.

## LACTIC FERMENT GROUP.

Bact. lacticum type.

2. Liquefy gelatin.

## GLANDERS GROUP.

Bact. mallei type.

3. Do not grow well on gelatin at room-temperature.

A. Stain with basic aniline dyes, but are easily decolorized by mineral acids when stained with carbol-fuchsin.

- (a) Grow well in bouillon at body-temperature and stain by Gram's method.

## DIPHTHERIA GROUP.

Bact. diphtheriæ type.

- (b) Do not grow in bouillon or on ordinary media.

1. Rods slender.

a. Stain by Gram's method.

## LEPROSY GROUP.

Bact. lepræ type.

- b. Do not stain by Gram's method.

## INFLUENZA GROUP.

Bact. influenzæ type.

2. Rods variable.

## ROOT-TUBERCLE GROUP.

- B. Do not stain with aqueous solutions of basic anilins and are not easily decolorized by acids.

## TUBERCLE GROUP.

Bact. tuberculosis type.

## BACILLUS.

- I. Spore formers.

1. Aërobic and facultative anaërobic. Rods not swollen at sporulation.



A. Liquefy gelatin slowly.

UROBACILLUS GROUP OF MIQUEL.

B. Liquefy gelatin quickly.

(a) Potato cultures rugose.

POTATO-BACILLUS GROUP.

B. mesentericus type.

(b) Potato cultures smooth.

B. SUBTILIS GROUP.

B. subtilis type.

2. Obligate anaërobic.

A. Rods not swollen at sporulation.

MALIGNANT EDEMA GROUP.

B. chauvæi type.

B. Rods clavate at sporulation.

TETANUS GROUP.

B. tetani type.

II. Spore formation not observed.

I. Aërobic and facultative anaërobic.

A. Gelatin colonies roundish, not distinctly ameboid.

(a) Do not liquefy gelatin.

I. Do not stain by Gram's method.

a. Generate gas in glucose bouillon.

(1) Coagulate milk.

COLON GROUP.

B. coli type.

(2) Do not coagulate milk.

HOG-CHOLERA GROUP.

B. suipestifer type.

- b*. Do not generate gas in glucose bouillon.

TYPHOID GROUP.

*B. typhosus* type.

- 2. Stain by Gram's method.

*B. MURIPESTIFER* GROUP.

*B. muripestifer* type.

- (*b*) Liquefy gelatin and generate gas in glucose bouillon.

*B. CLOACÆ* GROUP.

*B. cloacæ* type.

- B.* Gelatin colonies ameboid or irregular.

- (*a*) Do not liquefy gelatin.

*B. ZOPFI* GROUP.

*B. zopfi* type.

- (*b*) Liquefy gelatin.

PROTEUS GROUP.

*B. vulgaris* type.

MICROSPIRA.

- I. Not phosphorescent.

- 1. Do not liquefy gelatin, or only slightly.

MSP. SAPROPHILES GROUP.

*Msp. saprophiles* type.

- 2. Liquefy gelatin.

- A. Produce indol.

- (*a*) Very pathogenic to pigeons.

MSP. METSCHNIKOWI GROUP.

*Msp. metschnikovi* type.

(b) Not distinctly pathogenic to pigeons.

**CHOLERA GROUP.**

Msp. comma type.

B. Do not produce indol, or very little, at least after twenty-four hours.

**CHOLERA-NOSTRAS GROUP.**

Msp. finkleri type.

**IV. CLASSIFICATION OF WATER BACTERIA BY GROUPS.**

Fuller and Johnson have applied the group method of classification to the bacteria of water as follows :<sup>1</sup>

**WATER BACTERIA.**

- |  |            |
|--|------------|
| I. Fluorescent.                                | Group I.   |
| II. Non-fluorescent.                           |            |
| 1. Chromogenic.                                |            |
| A. Red.  | Group II.  |
| B. Orange.                                     | Group III. |
| C. Yellow.                                     | Group IV.  |
| D. Violet.                                     | Group V.   |
| 2. Non-chromogenic.                            |            |
| A. Gelatin liquefied.                          |            |
| (a) Characteristic colonies on gelatin plates. |            |

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<sup>1</sup> *Jour. of Exp. Med.*, vol. iv., Nos. 5-6, p. 609. Conn has adopted a somewhat similar classification into groups for the dairy bacteria. See *Report of the Storrs* (Connecticut) *Agricultural Experiment Station for 1899*.

- 1. *Proteus* forms.           Group VI.
  - 2. *Subtilis* forms.       Group VII.
  - (*b*) Non-characteristic colonies on gelatin plates.
    - 1. Fermentation of carbohydrates.
      - a.* Gas production.   Group VIII.
      - b.* No gas production. Group IX.
    - 2. Non-fermentation of carbohydrates. Group X.
- B. Gelatin not liquefied.
- (*a*) Fermentation of carbohydrates.
    - 1. Gas production.   Group XI.
    - 2. No gas production. Group XII.
  - (*b*) Non-fermentation of carbohydrates.   Group XIII.

(a) Transfer 1 c.c. of the water to be examined, by means of a sterile graduated pipet, to each of three tubes of liquefied gelatin.<sup>1</sup> Use a sterile pipet for each transfer.

(b) Shake the tubes, flame the lips, and pour into sterile Petri dishes. Place these in the dark at 20° C.

(c) Examine the plates from day to day, and count the colonies that appear.

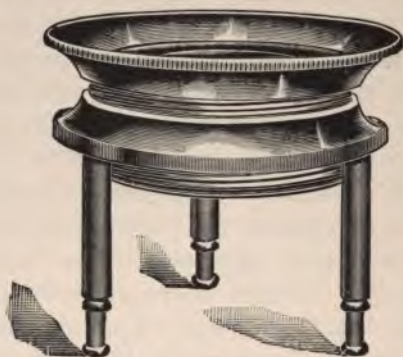


FIG. 50.—Simple microscope for counting colonies.

It is best to count all the colonies if possible ; but when they are very numerous, some one of the various methods devised for counting colonies must be employed.

(1) Wolfhügel's counting-plate consists of a glass plate on which are ruled square centimeters. The Petri dish is

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<sup>1</sup> If the water is suspected of containing large numbers of bacteria, smaller quantities than 1 c.c. should be added to each tube, or, better, dilute the water by the addition of a known quantity of sterile water. Many workers prefer to mix the water and the gelatin in the Petri dish instead of in the tube.

purposes. Water containing more than 500 per cubic centimeter should be looked upon with suspicion.

**2. Qualitative Analysis of Water.**—The qualitative examination of water requires not only the isolation of the several species present, but also their cultivation and the determination of their pathogenic or non-pathogenic properties. Such an examination takes a long time, and under most favorable circumstances it is very difficult to recognize the presence of pathogenic forms.

The principal value of the qualitative analysis of water is in the detection of contamination by sewage. Sewage is always liable to contain the evacuations of patients sick with typhoid fever or other transmissible diseases. The germs of typhoid fever are not easily identified, but there are certain bacteria common in human and other animal evacuations and in sewage (*B. coli*, *B. vulgaris*, *B. cloacæ*, *B. sporogenes*, *Bact. aërogenes*) whose presence is easily detected. Consequently the presence of such forms, though harmless in themselves, always indicates contamination.

**3. Laboratory Work in Water Analysis.**—All samples should be collected in sterile flasks, and cultures should be made immediately to secure accurate results. If transportation is necessary, the samples should be packed in ice. Tap water should be allowed to run a few minutes before the sample is taken ; if spring or well water is to be examined, the sample should be collected from about a foot below the surface.

(2) Colonies in circular dishes may be counted by means of Jeffer's plate. In this each circle is marked with its area in square centimeters, and each division equals 1 square centimeter.

(3) The colonies appearing under the microscope in the field of a low-power objective may be counted, in several parts of the dish, the average taken, and the number in the whole dish estimated by the equation :

$$\frac{\text{Number of colonies in the field}}{\text{Whole number of colonies}} = \frac{(\frac{1}{4} \text{ diam. of field})^2}{(\frac{1}{4} \text{ diam. of dish})^2}$$

(d) If a qualitative analysis is required, isolate and cultivate the different kinds of colonies.<sup>1</sup>

#### 4. Test for the Presence of *Bacillus coli*.—

(a) Prepare 10 fermentation-tubes of sterile bouillon containing 1 per cent. glucose.

(b) To each tube add 1 c.c. of the water to be tested.

(c) Place the tubes in the incubator at 37.5° C. for three days.

(d) Note the amount of gas which forms on each of the three days.

If gas-forming bacteria are present, gas will collect in the closed tube. The number of tubes showing the presence of gas gives a rough idea of the number of gas-producing bacilli present.

*Bacillus coli*, if present, will fill the closed tube by the second day. Too little or too much gas does not point to the presence of *Bacillus coli*. *Bacillus coli* forms most of its gas during the first twenty-four hours. The liquid in the bulb must

<sup>1</sup> See p. 112 for Fuller and Johnson's groups of water bacteria.

be distinctly acid to indicate the presence of *Bacillus coli*.

**5. Isolation of *Bacillus coli*.—First Method.—**

(a) Add 50 c.c. of the water to be tested to 50 c.c. of sterile bouillon in a sterile flask.

(b) Place in the incubator at 37.5° C. for two days.

The high temperature will destroy the common water bacteria, but will encourage the growth of the coli group.

(c) Test part of this culture for indol. Its presence indicates the presence of *Bacillus coli*.

(d) Make plates from this culture.

(e) If any non-liquefying colonies are whitish with irregular leafy outlines and show lines more or less radial, they are probably colonies of *Bacillus coli*, and must be carefully studied in cultures.<sup>1</sup>

**Second Method.**—(a) Add 70 c.c. of the water to be tested to 30 c.c. of sterile bouillon.

(b) To the mixture add 1 c.c. of 5 per cent. carbolic acid.

(c) Incubate at 37.5° C. for twenty-four hours. Carbolic acid restrains the growth of the ordinary water bacteria, while the coli group and other intestinal forms grow unhindered.

(d) From the growth that results inoculate fermentation-tubes containing 1 per cent. glucose bouillon. Note the amount of gas that forms as before.

(e) Make plates from the growth in the tubes,

---

<sup>1</sup> See page 60.



and study the colonies that resemble those of *Bacillus coli*.

**6. Milk Analysis.**—The analysis of milk is conducted in the same manner as is that of water, but on account of the great number of bacteria in



FIG. 53.—Hesse's apparatus for collecting bacteria from the air.

milk the samples must be diluted with sterile water.

*Bacillus typhosus* and *Bacillus coli* are detected in milk by the same methods as in water analysis. Tuberculosis bacilli may be detected in milk by the method described in the next chapter.<sup>1</sup>

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<sup>1</sup> See page 157.

For the classification of bacteria in milk, see H. W. Conn, *Report of the Storrs (Connecticut) Agricultural Experiment Station for 1899*.



FIG. 54.—Petri's sand-filter for air examination.



FIG. 55.—Sedgwick's expanded tube for air examination.

**7. Bacteria in the Air.**—(a) Prepare 3 gelatin plates and expose them to the air for four or five

minutes in different places. (In recitation-rooms before and after class, out of doors, etc.)

(b) Cover and allow to grow.

(c) Examine from day to day, and make cultures from the different colonies.

This is a rough method of determining the relative number of bacteria in the air. For more exact results recourse must be had to special apparatus for aspirating through bouillon or through sugar, as described in the text-books.

**8. Bacteria in the Soil.**—Numerous species of



FIG. 56.—Fränkel's instrument for obtaining earth from various depths for bacteriologic examination.

bacteria occur in the soil; some are of special interest on account of their pathogenic properties. Many are anaërobic, and this fact must be kept in mind while studying them.

To determine the number of bacteria in a sample of soil :

(a) Collect the soil without contamination from bacteria from other sources.

(b) Introduce a measured quantity into a tube of liquefied gelatin. Crush with a platinum needle, and mix thoroughly with the medium.

(c) Make plates, count, isolate, and study in the usual way.

Another method, and one which does away with the presence of particles of soil in the medium, but which perhaps does not give such accurate results, is to mix the sample thoroughly with sterile water and then make plates from the water.

## CHAPTER X.

### PATHOGENIC BACTERIA.

**THE** methods for the study of pathogenic bacteria are exactly the same as those already described. In certain cases only are special culture-media necessary for their growth. Most pathogenic forms grow better in the incubator at body-temperature,  $37.5^{\circ}$  C. In all cases animal inoculations are necessary for the determination of pathogenicity.

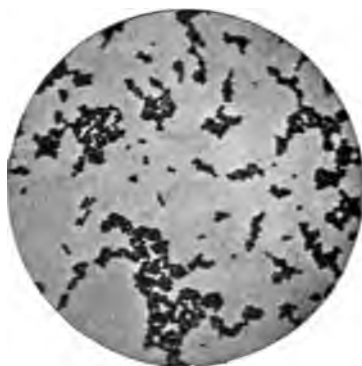


FIG. 57.—*Micrococcus aureus*, from an agar-agar culture (Günther).

Great care must be used in handling pathogenic cultures in order to avoid accidents. Have at hand a solution of corrosive sublimate (1 : 1000) or carbolic acid (1 : 20), with which to flood any material that

may by accident be spilled on floor or table. Carefully sterilize everything that the pathogenic material may have contaminated. Thoroughly disinfect the hands, instruments, and table with the corrosive sublimate solution after completing the work.



FIG. 58.—*Streptococcus pyogenes* (from a bouillon culture).

### I. PYOGENIC ORGANISMS.

(a) Study, according to the schedule in Chapter VIII., the morphology and biology of :

- Micrococcus aureus* (*Staphylococcus pyogenes aureus*).
- Micrococcus citreus* (*Staphylococcus pyogenes citreus*).
- Micrococcus pyogenes* (*Staphylococcus pyogenes albus*).
- Streptococcus pyogenes*.
- Sarcina tetragena* (*Micrococcus tetragenus*).

(b) Select and weigh a well-grown rabbit. Inoculate into the ear vein by means of a hypodermic syringe<sup>1</sup> 1 c.c. of a twenty-four or forty-eight hour bouillon culture of one of the above species, or of pus taken directly from an abscess.

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<sup>1</sup> Keep the syringe for some time before the operation in a 2 per cent. solution of carbolic acid. Wash it out five or six times with sterile water or bouillon before the inoculation ; or the syringe and needle may be boiled for five minutes before using.

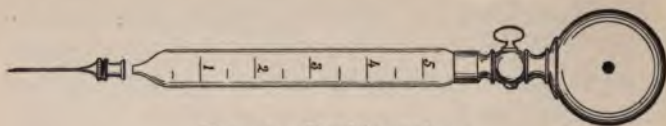


FIG. 59.—Koch's syringe.

(c) Record the daily weight of the animal until death.

(d) Perform an autopsy on the dead rabbit, note



FIG. 60.—Method of making an intravenous injection into a rabbit. Observe that the needle enters the posterior vein from the hairy surface (McFarland).

the various pathologic changes, and make cultures and cover-glass smears from all the organs and from the blood.<sup>1</sup>

<sup>1</sup>Sear the surface of the body-wall and of each viscus with a red-hot spatula or old scalpel before cutting into

(e) Preserve some of tissues in absolute alcohol or in Zenker's fluid.<sup>1</sup>

(f) Stain the smear preparations and examine for the inoculated organism.

(g) Make pure cultures from whatever growth is

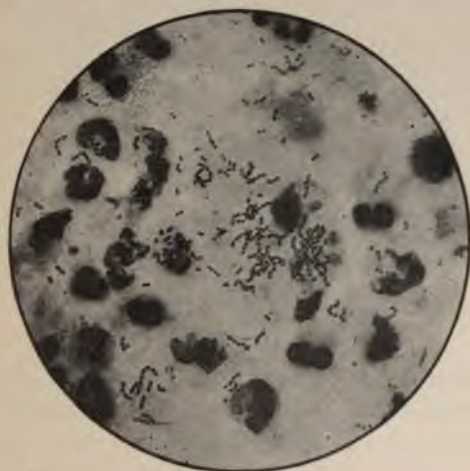


FIG. 61.—*Streptococcus pyogenes*; cover-glass preparation from the pus of an abscess;  $\times 1000$  (Fränkel and Pfeiffer).

obtained in the cultures from the blood and organs, and try to recover the original organism.

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them; sterilize the blades of the knives, scissors, and forceps used in making the incisions by dipping them in methyl alcohol and passing them quickly near enough to a Bunsen flame to ignite the alcohol. Smears and cultures from the blood are best made from the blood in the heart. For the method of preparation of blood-smears, see page 59.

<sup>1</sup> See Appendix, page 173.





FIG. 62.—*Streptococcus pyogenes*, seen in a section through human skin ;  $\times 500$  (Fränkel and Pfeiffer).

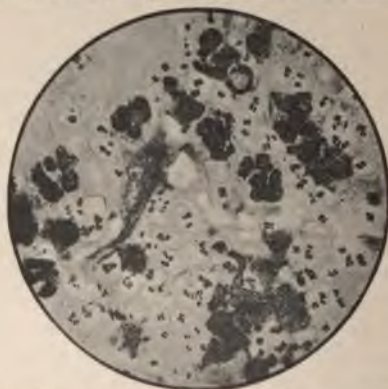


FIG. 63.—*Sarcina tetragena* in pus from a white mouse ;  $\times 615$  (Heim).

(h) Section the preserved tissue and stain for bacteria with carbol-thionin-blue, Kuhne's methylene-blue, or by Gram's method.

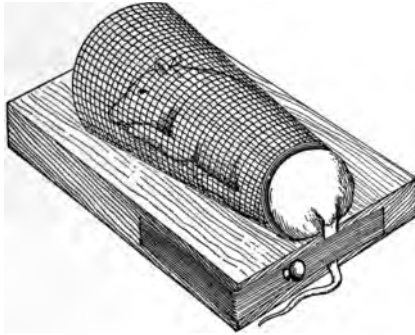


FIG. 64.—Mouse-holder, with mouse in position for inoculation.

Other animals—guinea-pigs, rats, or mice—may be used for inoculations. Inoculations may be subcutaneous, peritoneal, or intravenous, according to the organisms used or the nature of the experiment.

## II. GONOCOCCUS.

1. Examine cultures of *Micrococcus gonorrhææ*, and stain the organism with Löffler's blue and by Gram's method.

2. Examine gonorrheal pus as follows :

- (a) Prepare films on cover-glasses.
- (b) Pass three times through flame.
- (c) Stain with Löffler's methylene-blue or with any aqueous anilin stain one minute.
- (d) Wash, dry, and mount.
- (e) Examine with the oil-immersion lens.

Gonococci are of medium size, composed usually of two hemispheres separated by a narrow unstained interval. Occasionally two pairs of cocci form a "tetrad." The cocci are usually within the leukocytes.

(f) Stain another film by Gram's method. The gonococci are decolorized.

### III. ANTHRAX.

1. Study the morphology and biology of cultures of *Bacterium anthracis*.
2. Inoculate a guinea-pig as follows :

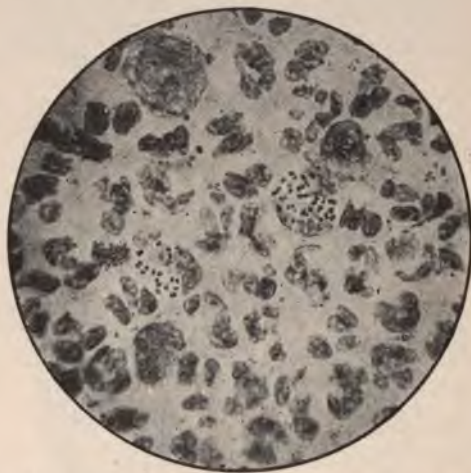


FIG. 65.—Gonococcus in urethral pus;  $\times 1000$  (Fränkel and Pfeiffer).

- (a) Remove the hair from a small area on the abdomen.

(b) With a snip of a pair of sterile scissors make a little subcutaneous pocket.

(c) Introduce into this pocket spores and bacteria from a pure culture by means of a platinum loop.

(a) At the autopsy prepare cultures, smears, and sections from the organs.



FIG. 66.—*Bacterium anthracis*; colony three days old upon a gelatin plate; adhesive preparation;  $\times 1000$  (Fränkel and Pfeiffer).

(e) Make cover-glass preparations from the blood as follows :

1. Place a small drop of blood between two absolutely clean cover-glasses, draw them apart and allow the smears to dry.

2. Fix in equal parts of ether and alcohol for thirty minutes, or in absolute alcohol five minutes,

or in vapor of formaldehyd two and a half minutes; or heat in the thermostat at  $110^{\circ}$ – $120^{\circ}$  C. for twelve hours; or heat on a brass plate for one hour at the point where water boils.



FIG. 67.—*Bacterium anthracis*; cover-glass preparation from the spleen of a mouse (Mallory and Wright).

3. Stain in eosin ( $\frac{1}{2}$  per cent. in 60 per cent. alcohol) from one to five minutes.
4. Wash in water and dry.
5. Contrast-stain in aqueous methylene-blue from one-half to one minute.
6. Wash, dry, and mount.



## IV. GLANDERS.

1. Study the morphology and biology of the glanders bacterium.
2. Inoculate a male guinea-pig intraperitoneally with 1 c.c. of a bouillon culture.
3. Note in two or three days the great swelling

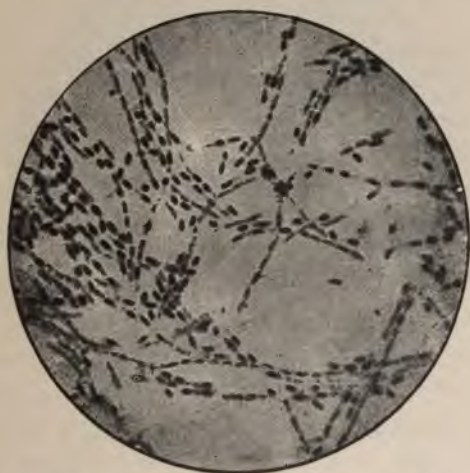


FIG. 68.—*Bacterium anthracis*, stained to show spores;  
× 1000 (Fränkel and Pfeiffer).

and redness of the testicles, caused by a semipurulent affection of the tunica vaginalis. This is a diagnostic test for the glanders bacterium.

4. At the autopsy prepare smears, cultures, and sections from all the organs and from the peritoneal and scrotal nodules.

5. Stain sections as follows :

- (a) Carbol-thionin-blue for ten to fifteen minutes.
- (b) Wash thoroughly in water.
- (c) Dehydrate in anilin oil.
- (d) Treat with equal parts of anilin oil and xylol.
- (e) Pass through pure xylol to balsam.

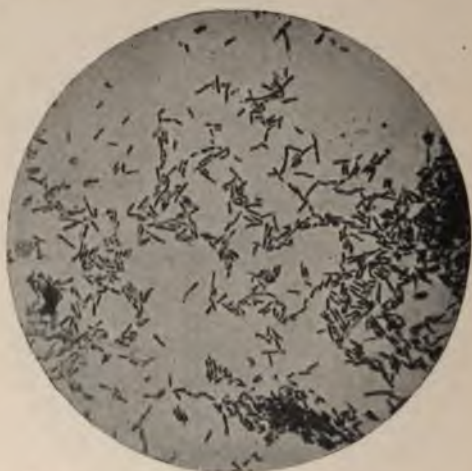


FIG. 69.—*Bacterium mallei*, from a culture upon glycerin agar ;  $\times 1000$  (Fränkel and Pfeiffer).

6. For the diagnosis of a suspected case of glanders proceed as follows :

(a) Rub a large swab made of absorbent cotton in the discharge from the nose or in the suspected ulcer.

(b) Transfer the swab to 5 c.c. of sterile water and shake thoroughly.

(c) Inoculate the resulting suspension intraperitoneally into a well-grown male guinea-pig.

(*d*) In two to seven days scrotal inflammation will develop if the glanders bacterium was present. If the glanders bacterium was not present, after a few hours or days of depression the guinea-pig recovers completely. It may happen that some acute septic organisms were present in the material injected. In such cases the guinea-pig usually dies within twenty-four hours, and the test is evidently of no value and must be repeated.

(*e*) If the scrotal lesions appear, perform an autopsy. Look particularly for nodular deposits in the peritoneum and visceral layer of the tunica vaginalis, infiltration of the scrotal tissue, and edema extending into the groin and suprapubic region.

(*f*) Transfer aseptically a portion of a nodule to potato, and place at  $37.5^{\circ}$  C. In twenty-four to forty-eight hours small, smooth, glistening, amber-colored colonies of the glanders bacterium should develop. The bacterium is a short, thick rod, with rounded ends, usually slightly curved or bent, sometimes elongated into threads. It stains faintly in Löffler's methylene-blue.

(*g*) A positive diagnosis can usually be made from the gross lesions, but the isolation of the organism makes the diagnosis certain.

## V. DIPHTHERIA.

1. Make swabbings from the throats of healthy individuals and from several diphtheria patients in hospital. Depress the tongue and rub a sterile





FIG. 70.

FIG. 70.—Providence Health Department outfit for diphtheria diagnosis. A pasteboard box containing a swab-tube and a serum-tube, both with etched surface on which to write the name and address of patients, etc.

FIG. 71.

FIG. 71.—*Bacterium diphtheriae*; agar culture (photograph by Dr. Henry Koplik).

swab made of non-absorbent cotton over the back of the throat, tonsils, diphtheritic membrane, etc.

3. Rub the swab over the surface of a Löffler blood-serum tube.

3. Place the tube in an incubator at  $37.5^{\circ}$  C. for sixteen hours or longer.
4. Examine the growth for the small grayish, slightly elevated diphtheria colonies.
5. Prepare films from a suspected colony.
6. Pass three times through the flame and stain in Löffler's methylene-blue for one minute.



FIG. 72.—*Bacterium diphtheriae*, from culture upon blood-serum ;  $\times 1000$  (Fränkel and Pfeiffer).

7. Wash, dry, mount, and examine with the  $\frac{1}{12}$  inch oil-immersion lens. The characteristic diphtheria bacteria can easily be detected.
8. Isolate the diphtheria organisms in pure culture by inoculating tubes from a single colony which on examination proves to be diphtheria. If a single colony cannot be found, touch the needle

once to the growth on the serum-tube and make a series of strokes on 3 or 4 tubes. When the colonies develop, those in the last tube will be sufficiently distinct so that pure cultures may be made from them. Another method is to inoculate a bouillon-tube or a tube of sterile water or normal salt solution, and immediately from this make stroke cult-



FIG. 73.—*Bacterium diphtheriae*, colony twenty-four hours old upon agar;  $\times 100$  (Fränkel and Pfeiffer).

ures on serum-tubes. The growth on these will probably be in individual colonies. Either of these methods obviates the necessity of making plates.

9. Study the morphology and biology of the diphtheria bacterium obtained above.

10. The following stains are diagnostic for the diphtheria bacterium :

*Hunt's Stain.*

- (a) Prepare films as usual.
- (b) Stain in aqueous methylene-blue for one minute.
- (c) Wash in water and dry.
- (d) Treat with a 10 per cent. solution of tannic acid for one minute.
- (e) Wash in water and dry.
- (f) Stain in aqueous methyl-orange for one minute.
- (g) Wash, dry, and mount.

*Neisser's Stain.*

- (a) Prepare solution A as follows :

Methylene-blue,	1 gm.;
Alcohol (95 per cent.),	20 c.c.

Dissolve and add

Acetic acid,	50 c.c.;
Water,	950 c.c.

- (b) Prepare solution B as follows:

Bismarck-brown,	2 gm.;
Boiling water,	1000 c.c.

- (c) Treat films with solution A for one to three seconds.

- (d) Wash in water.
- (e) Treat with solution B for three to five seconds.
- (f) Wash, dry, and mount.

II. Test the virulence of the diphtheria organism isolated as follows :

(a) Prepare a twenty-four to forty-eight hour bouillon culture.

(b) Sterilize a hypodermic syringe and needle by soaking in 2 per cent. carbolic acid and washing out in sterile water or bouillon, or by boiling for five minutes.

(c) Select and weigh a full-grown guinea-pig.

(d) While the pig is held on its back on the table



FIG. 74.—Slide, 7 x 2½ inches, for the routine examination of diphtheria cultures. Each square can be placed under the lens of the microscope without disturbing the equilibrium of the slide.

by an assistant, the hair is removed from a small area on the ventral abdominal wall.

(e) With the thumb and forefinger of the left hand pinch up a fold of the skin, and with the right hand insert the hypodermic needle between the skin and the muscular body-wall.

(f) Inoculate an amount of the culture equal to 1 per cent. of the weight of the pig.

(g) Record the daily weight of the pig until death.

(h) Perform an autopsy on the dead pig ; note the pathologic changes, and prepare cultures, smears, and sections from the organs and from the point of inoculation.

## VI. INFLUENZA.

1. Prepare blood-agar tubes by smearing the surface of a ordinary agar-tube with a drop of blood

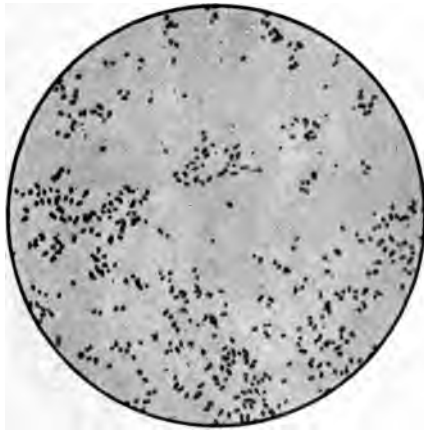


FIG. 75.—*Bacterium influenzae*, from a gelatin culture ;  
× 1000 (Itzerott and Niemann).

obtained aseptically from man, rabbit, guinea-pig, pigeon, or frog.

2. Break up a distinctly purulent portion of influenza sputum in 1 or 2 c.c. of bouillon, and spread a loopful of the suspension over the surface of the blood-agar tube.

3. Place in the incubator and examine at the end of from eighteen to twenty-four hours.

4. The influenza colonies appear as minute colorless, glassy, transparent points, resembling drops of dew. They are barely visible to the unpractised eye, and require a low magnifying power to be seen clearly.

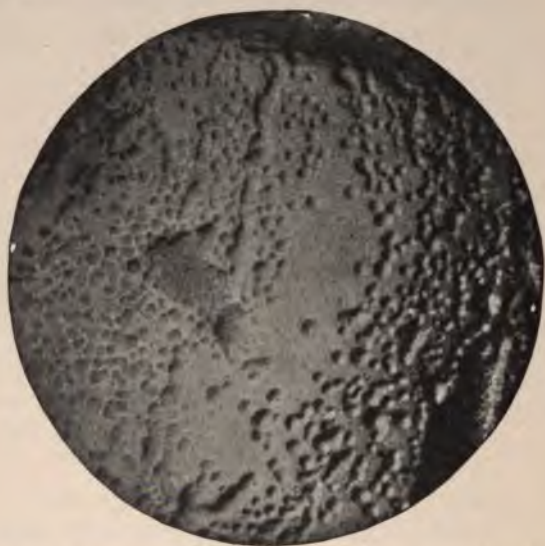


FIG. 76.—*Bacterium influenzae*: colonies on blood-agar; low magnifying power (Pfeiffer).

5. Study the morphology and biology of the organisms in one of these colonies. They should not grow on ordinary media, and should have the morphology of the influenza bacteria.

6. Prepare smears from one of the purulent



masses in the sputum. Stain in very dilute carbol-fuchsin for five to ten minutes, or in Löffler's methylene-blue heated to the steaming-point. The influenza bacteria are very small, short, with

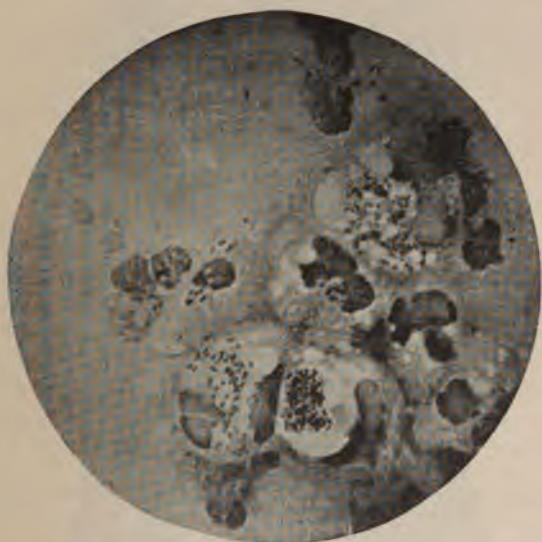


FIG. 77.—*Bacterium influenzae*: cover-glass preparation of sputum from a case of influenza, showing the bacteria within the leucocytes; highly magnified (Pfeiffer).

round ends, are often present in large numbers, and are frequently within the pus cells. They may occur in pairs, and then resemble cocci. The ends are usually more deeply stained than the central portions.



## VIB. TYPHOSUS AND COLON BACILLI.



*Vib. typhosus* (typical form) in a healthy human fecal culture.  $\times 1000$  (Hesse).



*Vib. typhosus* (typical form) in a healthy human fecal culture.  $\times 1000$  (Hesse).

\* Study the morphology and biology of cultures

of *Bacillus typhosus* and *Bacillus coli*. Some of the differences between them are indicated below :

B. TYPHOSUS.	B. COLI.
Rods usually slender.	Rod inclined to be a little thicker.
Flagella more numerous, longer, more wavy (10-20).	Flagella fewer and shorter (8-10).
In artificial media growth generally slower and not so vigorous.	Growth faster and more vigorous.
Growth on fresh acid potato a nearly transparent film.	Growth on potato a brownish pellicle.
Very slight acid production in ordinary media, followed sometimes by a production of alkali.	Well-marked acid production.
Litmus milk—no change.	Litmus milk—pink color.
Milk not coagulated.	Milk coagulated.
Fermentation of lactose very slight if any.	Fermentation of lactose pronounced.
Litmus lactose agar—no change in color.	Litmus lactose agar—production of red color.
Glucose media—no gas formation.	Abundant gas formation.
No production of indol in ordinary bouillon.	Well-marked indol production in ordinary bouillon.
Does not grow in Maassen's asparagin-glycerin solution.	Grows in Maassen's solution.
Agglutination-test positive.	Agglutination-test usually negative.

2. Study sections of spleen, intestinal lesions, mesenteric lymph-glands, etc., from a human typhoid autopsy. Stain with carbol-thionin-blue or carbol-fuchsin.

3. **Widal Reaction in Typhoid Fever.**—If the blood-serum of a person suffering with typhoid fever or of one who has recently recovered from it

be added to a bouillon culture of actively motile typhoid bacilli, the bacilli lose their motility and



FIG. 58.—*Bacillus typhosus*: superficial colony two days old on a gelatin plate;  $\times 20$  (Heim).

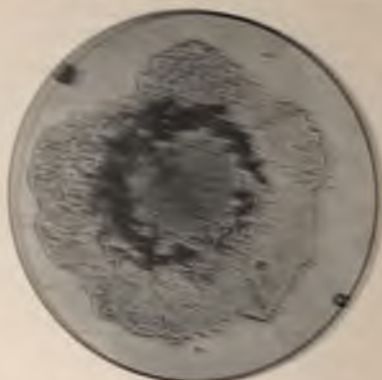


FIG. 59.—*Bacillus coli*: superficial colony two days old, on a gelatin plate;  $\times 20$  (Heim).

soon aggregate in clumps. Dilutions of serum 1:10 and 1:30 with a half-hour time-limit are

those most commonly employed. In doubtful cases a dilution of 1 : 100 with a one-hour time-limit is recommended.

(a) Place 9 drops of a twenty-four-hour bouillon culture of actively motile typhoid bacilli on separate



FIG. 82.—*Bacillus typhosus*, from an agar culture six hours old, showing the flagella stained by Löffler's method ;  $\times 1000$  (Fränkel and Pfeiffer).

spots on a clean cover-glass. Add 1 drop of serum from the blood of the suspected typhoid case,<sup>1</sup> mix

<sup>1</sup> The blood may be collected in a capillary tube and after coagulation the serum removed; or dried blood may be moistened with water and the resulting solution used as serum. The latter method, however, does not permit accurate dilution. Chester and Robin have recently devised a pipet for delivering a measured drop of blood, so that dilu-

all together, and mount as a hanging-drop preparation.<sup>1</sup>

(b) Examine with the  $\frac{1}{6}$  inch or with the oil-

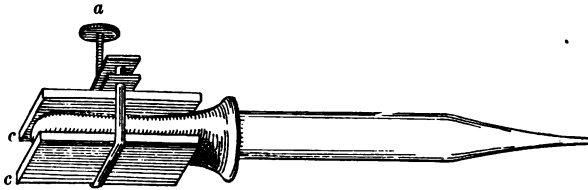


FIG. 83.—The Chester and Robin pipet for delivering uniform drops of blood for the Widal test. A glass and rubber pipet, the bulb of which is enclosed between two strips of metal held in place by a Hoffman clamp.

immersion lens. If the case is one of typhoid, after some time, varying between a few seconds and a

tions may be made with a fair degree of accuracy from dried blood. It consists of an ordinary medicine-dropper of a given size, the bulb of which is enclosed on either side by two narrow strips of metal (Fig. 83, *c, c.*), and both placed in a medium-sized Hoffman clamp. The inward movement of the clamp by means of the screw *a* compresses the bulb, while a slight turn in the opposite direction dilates it a little and permits a small drop of blood to enter. In expelling the blood the dropper is held vertically over a strip of thick filter-paper, and the clamp is slowly compressed until a single drop falls of its own weight. This drop is then dried, and when the test is to be applied the blood-spot is cut from the paper and drops of the diluting fluid are added from an exactly similar pipet until the required dilution is reached.

<sup>1</sup> It is convenient to use a slide on which two glass rings have been cemented, so that one may be used as a control, containing the culture of bacilli alone.

half-hour, the bacilli will be seen to become less motile, and finally to cease all movement and



FIG. 84.—Slide with two cells for observing Widal reaction with a control.

appear in clumps here and there throughout the field.

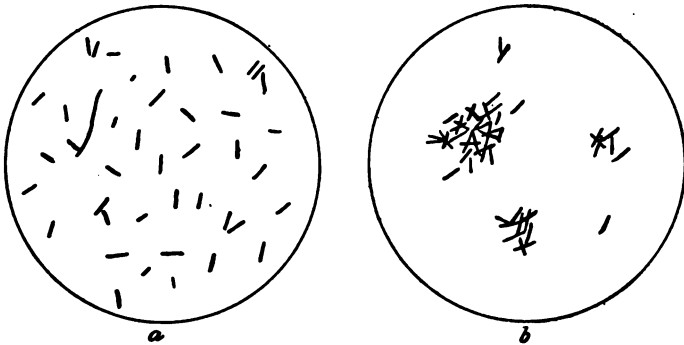


FIG. 85.—Widal reaction: *a*, bouillon culture of *Bacillus typhosus*; *b*, the same after the addition of typhoid serum.

This reaction can be reversed and made to serve as a means of identifying the typhoid bacillus if the serum of a person known to have typhoid fever is at hand.

## VIII. PNEUMONIA.

BACTERIUM PNEUMONIÆ (FRANKEL'S PNEUMOCOCCUS)  
AND BACTERIUM PNEUMONICUM (FRIEDLANDER'S  
PNEUMOBACILLUS).

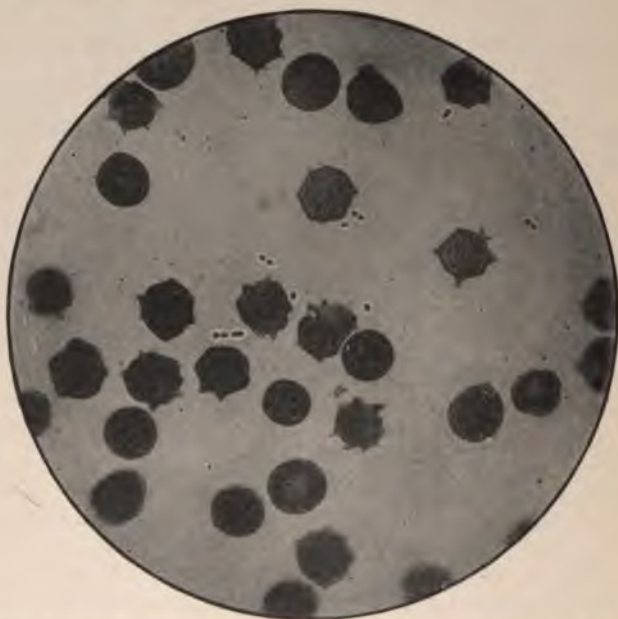


FIG. 86.—*Bacterium pneumoniae*, from the heart's blood of a rabbit;  $\times 1000$  (Fränkel and Pfeiffer).

1. Study the morphology and biology of cultures of *B. pneumoniae* and *B. pneumonicum*.
2. Make cover-glass preparations from pneumonia sputum. Stain in Löffler's methylene-blue or carbol-fuchsin, diluted one-half.
3. Prepare sections of lung-tissue from cases of



lobar pneumonia, general infection, etc. Stain in carbol-thionin-blue and by Gram's method.

4. Inoculate a guinea-pig or rabbit subcutaneously or intravenously with virulent cultures or with



FIG. 87.—*Bacterium pneumonicum*, from the expectoration of a pneumonia patient;  $\times 1000$  (Fränkel and Pfeiffer).

pneumonia sputum; also with sputum from healthy persons.

5. At the autopsy note the pathologic changes, prepare smears from the blood and organs, make cultures, and prepare sections.

## IX. TUBERCULOSIS.

### 1. Examination of Cultures.

Study the morphology and biology of *Bacterium tuberculosis*.



(1) For cultures use blood-serum, agar, and bouillon to which from 4 to 8 per cent. of glycerin has been added.

(2) Stain films, prepared from cultures, with ordinary stains<sup>1</sup> and by the Ziehl-Neelson method as follows :

(a) Prepare films as usual.

(b) Stain in Ziehl's carbol-fuchsin, steaming but not boiling, five minutes ; cold, twenty minutes.

(c) Wash in 20 per cent. sulphuric acid for three to five seconds.

(d) Wash in 60 per cent. alcohol until no red color is left.

(e) Wash in water.

(f) Stain in aqueous solution of methylene-blue for one minute.

(g) Wash in water, dry, and mount.

The bacteria of leprosy, syphilis, and smegma stain by this method. *Bacterium lepræ* stains much more quickly than *B. tuberculosis*, *B. syphilidis* is decolorized more quickly, especially in sulphuric acid, and *B. smegmatis* is decolorized by the alcohol.

## **2. Examination of Tuberculous Sputum.**

(1) **Ziehl-Neelson Method.**

(a) Place the sputum in a shallow glass dish on a black surface.

(b) Select several of the characteristic yellowish

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<sup>1</sup> The tubercle bacillus takes the ordinary stains very slowly and faintly.

particles, place between two cover-glasses, and spread evenly.

(c) Draw the covers apart and allow the films to dry.

(d) Pass three times through flame.



FIG. 88.—Tubercle bacteria in sputum (carbol-fuchsin and methylene-blue).

(e) Stain in hot (steaming but not boiling) Ziehl's carbol-fuchsin for five minutes or in the cold solution for twenty minutes.

(f) Wash rapidly in water.

(g) Decolorize in 20 per cent. sulphuric, hydrochloric, or nitric acid, for three to five seconds.

(*h*) Wash in water.

(*i*) Contrast-stain in saturated aqueous solution of methylene-blue or in Löffler's blue for one-half minute.

(*j*) Wash in water, dry, and mount.

(*k*) Examine with the  $\frac{1}{12}$  inch oil-immersion lens. The bacteria should be red in a blue field.<sup>1</sup>

(2) **Koch-Ehrlich Method.**

(*a*) Stain cover-glass preparations in anilin fuchsin or anilin gentian-violet for twelve to twenty-four hours.

(*b*) Decolorize for three to five seconds in 20 per cent. nitric acid.

(*c*) Wash in water, in 60 per cent. alcohol for five to ten seconds, and again in water.

(*d*) Contrast-stain in aqueous methylene-blue for one minute.

(*e*) Wash, dry, and mount.

(3) **Gabbet's Method.**

(*a*) Stain in steaming Ziehl's carbol-fuchsin for one minute.

(*b*) Wash in water for two to three seconds.

(*c*) Stain in Gabbet's blue<sup>2</sup> for thirty seconds or longer.

(*d*) Wash in water, dry, and mount.

(4) **Rosenberger's Method.**

(*a*) Stain in carbol-fuchsin (cold) for five to ten minutes.

(*b*) Without washing stain for one to two minutes

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<sup>1</sup> For the detection of tubercle bacteria in sputum when present in very small numbers see page 157.

<sup>2</sup> See Appendix, page 172.

in sweet spirits of nitre to which has been added enough alcoholic solution of malachite-green, Bismarck-brown, or methylene-blue to give a deep-colored fluid.

(c) Wash in water, dry, and mount.

### 3. Inoculations.

(a) Inoculate a guinea-pig subcutaneously in the abdominal wall with tuberculous material.

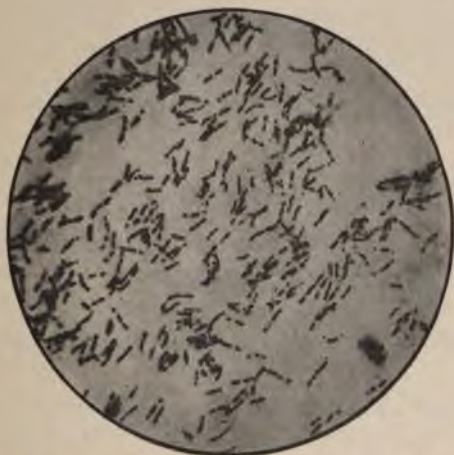


FIG. 89.—Tubercle bacteria in sputum (Fränkel and Pfeiffer).

(b) After four to six weeks,<sup>1</sup> when the inguinal lymphatic glands have become enlarged, kill the animal.

(c) With proper aseptic precautions make three or four cultures on blood-serum from two or three

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<sup>1</sup> For class-work such an animal must be previously inoculated.

glands, spreading a large quantity of the material on the surface of the tubes.

(*d*) Seal air-tight, place in the incubator, and examine the growth that occurs.

#### **4. Sections.**

(*a*) Prepare sections of tubercular lesions from the inoculated animals or from the human lung in acute phthisis.

(*b*) Stain by the Ziehl-Neelson, Koch-Ehrlich, or Rosenberger methods.

#### **5. Detection of Tubercle Bacteria in Urine.**

(*a*) Make smears from the deepest layer of sediment thrown down by the centrifuge, or in the sedimenting glass.

(*b*) Stain in hot (steaming but not boiling) Ziehl's carbol-fuchsin for one minute.

(*c*) Wash in water.

(*d*) Decolorize in 20 per cent. sulphuric acid until pink.

(*e*) Wash in water, 95 per cent. alcohol for thirty seconds, and again in water.

(*f*) Stain in Löffler's methylene-blue for twenty seconds.

(*g*) Wash in water, dry, and mount.

The smegma bacillus, which frequently occurs in urine, does not stain by this method, as it is decolorized by alcohol. Rosenberger's method also gives excellent results with urine sediment.

**6. Detection of Tubercle Bacteria in Milk.****(1) First Method.<sup>1</sup>**

(a) To 50 c.c. of suspected milk add 10 c.c. of carbolic acid.

(b) Shake vigorously for two to five minutes. Pour into a sedimenting glass, cover, and allow to stand for twenty-four hours ; or use a centrifuge.

(c) With a pipet remove the deepest layer of sediment and prepare films.

(d) Dry and pass three times through flame.

(e) Pass through equal parts of ether and alcohol.

(f) Dry and pass three times through the flame, and stain as directed for sputum.

(g) Mount and examine with the  $\frac{1}{12}$  inch oil-immersion lens.

**(2) Second Method.**

(a) To 20 c.c. of the milk add 1 c.c. of a 50 per cent. potash solution.

(b) Heat in boiling water until the mixture turns yellowish brown.

(c) Add 20 c.c. of acetic acid, shake, heat again for three minutes, and centrifuge.

(d) Wash the sediment with hot water, again centrifuge, and make cover-glass preparations from the second sediment.

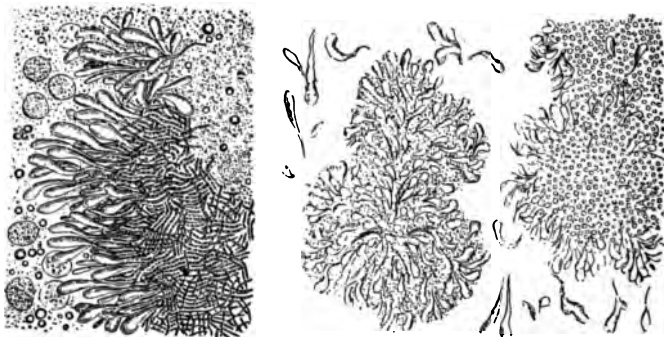
(e) Stain as directed for sputum.

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<sup>1</sup>This method may also be applied to the detection of tubercle bacteria in sputum when present in very small numbers, using 10-15 c.c. of sputum, 10 c.c. of water, and 6 c.c. of carbolic acid.

## X. ACTINOMYCOSIS.

1. Study the morphology and biology of cultures of *Actinomyces bovis*.
2. Prepare sections from tissue containing colonies of the parasite.
3. Stain by Gram's method or as follows :

FIG. 90.—*Actinomyces* (von Jaksch).

- (a) Stain deeply in saturated aqueous eosin solution for ten minutes.
- (b) Wash in water.
- (c) Stain in anilin-gentian-violet for two to five minutes.
- (d) Wash in normal salt solution.<sup>1</sup>
- (e) Treat with iodine solution (iodine, 1 part ; potassium iodid, 2 parts ; distilled water, 100 parts), for one minute.
- (f) Wash in water and dry slightly with filter-paper.

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<sup>1</sup> See Appendix, page 173.

- (g) Clear in anilin oil, then in several changes of xylol.
- (h) Mount in balsam.

## XI. MALARIA.

### 1. Examination of Fresh Blood for the Malarial Hematozoon.

- (a) Clean a slide and cover-glass.
- (b) Place a small drop of the blood to be examined on the slide, cover, and seal with vaselin.
- (c) Examine with the oil-immersion lens.

**The ameboid movements of the Hematozoon malariae** are visible at the room-temperature, but become more active if the slide is warmed.

### 2. Stained Preparations.

#### (1) First Method.

(a) Place a small drop of blood between two cover-glasses, slide the covers apart, and allow the films to dry.

(b) Fix by placing in absolute alcohol for five minutes or in the vapor of formaldehyd for two and a half minutes, or by one of the methods given under Anthrax (page 131).

(c) Stain in a 1 per cent. aqueous solution of eosin for five minutes or, if formaldehyd is used for fixation, in an alcoholic (60-75 per cent.) solution of eosin for three to ten minutes.

(d) Contrast-stain in saturated aqueous solution of methylene-blue for five minutes for the alcoholic preparations, for one minute for the formaldehyd



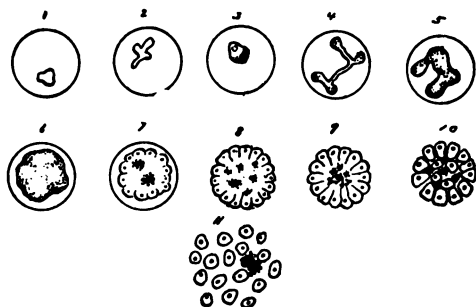


FIG. 91.—Some of the principal forms assumed by the Hematozoon of tertian fever in the course of its cycle of development (after Thayer and Hewetson).

preparations, or in Ehrlich-Biondi fluid<sup>1</sup> for one-half hour.

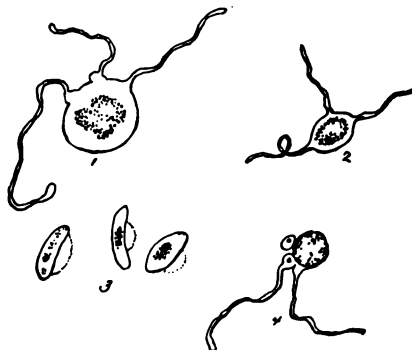


FIG. 92.—Crescentic and flagellated forms of Hematozoon malariae: 1, flagellated form of tertian fever; 2, flagellated form of quartan fever; 3, crescents, and 4, flagellated form of estivo-autumnal fever (after Thayer and Hewetson).

<sup>1</sup> See Appendix, page 173.

(e) Wash, dry, and mount. Ehrlich-Biondi preparations are examined dry.

In these preparations the red corpuscles are stained red, the malarial organisms and the nuclei of the leukocytes blue.

(2) **Whitney's Method.**

(a) Spread the blood as usual ; dry thoroughly in the air or by gentle heat.

(b) Treat for twenty seconds with the following modification of Zenker's fluid :

Potassium bichromate,                      2 gm. ;

Sodium sulphate,                              1 gm. ;

Water,    100 c.c.

Saturate while warm with corrosive sublimate.

Add 5 per cent. of strong nitric acid at time of using.

(c) Wash in water, and dry with filter-paper.

(d) Cover with Ehrlich's triacid stain<sup>1</sup> for three minutes.

(e) Wash, dry, and mount.

Unna's polychrome methylene-blue<sup>2</sup> and the Chenzinsky-Plein eosin and methylene-blue<sup>3</sup> solution work well after this fixation, but not Löffler's methylene-blue.

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<sup>1</sup> See Appendix, page 172.

<sup>2</sup> See Appendix, page 172.

<sup>3</sup> See Appendix, page 173.



## APPENDIX.

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### I. BACTERIAL MEASUREMENTS BY PHOTOGRAPHY.

Wilson and Randolph<sup>1</sup> recommend the following procedure for accurately measuring bacteria :

(a) Prepare a drawing about four times the size of the desired negative by ruling with ink two sets of equidistant lines at right angles to each other, making every tenth line somewhat heavier than the others.

(b) Reduce this drawing by photography to such a size that the rulings are exactly 1 millimeter apart.

(c) Adjust the photographic apparatus so that an amplification of 1000 diameters is secured. Obtain this by measuring the image of a stage micrometer on the ground-glass screen.

(d) With this adjustment make a photomicrograph of the bacteria to be measured.

(e) Superimpose the print of the photomicrograph of the bacteria on the print of the scale, or

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<sup>1</sup> *Journal of Applied Microscopy*, vol. ii, No. 11, Nov., 1899.

microns. The reading is directly in micromillimeters.



FIG. 93.—Wilson and Randolph's method of measuring bacteria by photography.

## II. MOULDS AND YEASTS.

Moulds and yeasts frequently contaminate plate and tube cultures, and inasmuch as these growths are occasionally the cause of pathologic conditions,



FIG. 94.—*Saccharomyces cerevisiæ*.

it is advisable that the bacteriologist be acquainted with certain typical forms.

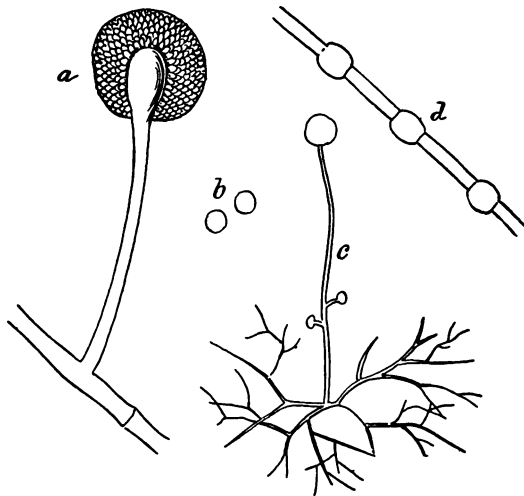


FIG. 95.—*Mucor racemosus*: *a*, spore-bearing head; *b*, spores; *c*, branch; *d*, resting spores (after Jelliffe).

**1. *Saccharomyces cerevisiæ*.**

Examine cultures of this yeast, mount, and stain exactly as directed for bacteria.

**2. *Mucor racemosus*.**

(1) Cultures of this mould may be made on the surface of gelatin- or agar-tubes, but preferably in plates. The developing colonies may be examined directly with the low-power lens of the microscope.

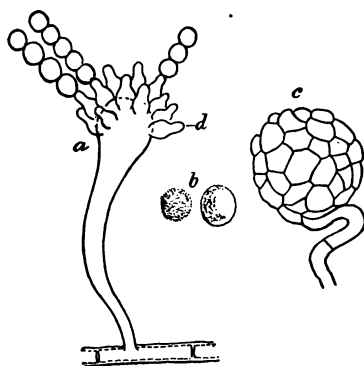


FIG. 96.—*Aspergillus repens*: *a*, conidia-bearing head; *b*, conidia; *c*, peritheca; *d*, sterigmata (after Jelliffe).

(2) For examination with the high power, remove a small portion of the growth to a mixture of glycerin and water on a slide; spread out as thin as possible, cover, and examine.

(3) The preparation may be stained by adding a little eosin solution to the glycerin and water.

(4) Permanent preparations may be made as follows:

(*a*) Transfer a small amount of the mould to a slide.

(b) Drop a little alcohol upon it to remove the air from the hyphæ.

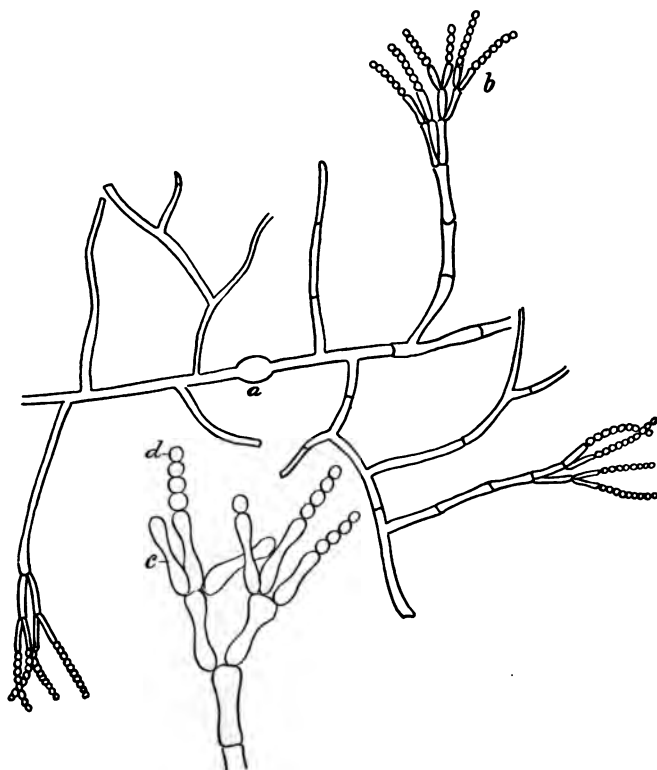


FIG. 97.—*Penicillium crustaceum* : *a*, conidia with mycelium ; *b*, conidia cluster ; *c*, sterigma ; *d*, conidia (after Jelliffe).

- (c) Treat again with water.
- (d) Stain in methylene-blue.
- (e) Mount in glycerin or glycerin-jelly.



### 3. *Aspergillus repens* and *Penicillium crustaceum*.

These moulds may be examined exactly as directed for *Mucor*.

### 4. Key for the Identification of the Yeasts and Moulds that most frequently contaminate Cultures.<sup>1</sup>

Mycelium growth absent:

#### I. YEASTS, SACCHAROMYCETACEÆ.

- I. Growth white to dirty white,  
*S. cerevisiæ.*  
*S. albicans.*
2. Growth reddish,  
*S. glutinis.*
3. Growth brownish to black,  
*S. niger.*

Mycelium growth present:

#### II. MOULDS.

- I. Spores inside of sporangia.

PHYCOMYCETES. Family, MUCORINI.

- (a) Mycelium of one kind,

- I. Spore-bearing bodies on unbranched hyphæ,

*Mucor.*

2. Spore-bearing bodies on branched hyphæ,

*Circinella.*

- (b) Mycelium of two kinds,

*Rhizopus.*

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<sup>1</sup>Adapted from "Some Laboratory Moulds," by S. E. Jelliffe, in *Journal of Pharmacology*, Nov., 1897.

## 2. Spores free, at the ends of modified hyphæ,

## HYPHOMYCETES.

## (a) Hyphæ pallid, loose, not collected into fascicles,

## MUCEDINEÆ.

## 1. Conidia undivided,

## a. Hyphæ short,

## (1) Hyphæ unbranched,

*Oospora.*

## (2) Hyphæ branched,

*Monilia.*

## b. Hyphæ elongated,

## (1) Conidia aggregated,

\* Fertile hyphæ enlarged at apex,

† Conidia on simple sterigmata.

*Aspergillus.*

†† Conidia on compound sterigmata,

*Sterigmatocystis.*

\*\* Fertile hyphæ not enlarged at apex,

*Penicillium.*

## (2) Conidia separated or loosely aggregated,

*Botrytis.*

## 2. Conidia once septate,

*Cephalothecium.*

## (b) Hyphæ brownish or black, not collected into fascicles,

## DEMATIÆ.

## 1. Conidia non-septate,

## a. Hyphæ short, only slightly different from conidia,

*Torula.*

b. Hyphae distinct from conidia,

*Hormodendron.*

2. Conidia septate,

a. Conidia in chains,

*Alternaria.*

b. Conidia single,

*Macrosporium.*

(c) Hyphae pallid or dark, collected into fascicles,

*STILBEE.*

(d) Hyphae pallid or reddish, collected in wart-like masses.

*TUBERCULARIE.*

Conidia elongated, septate,

*Fusarium.*

### III. STAINS AND REAGENTS USED IN THE STUDY OF BACTERIA.

#### Simple Anilin Stains.

Stock solutions of gentian-violet, methylene-blue, basic fuchsin, and crystal-violet are made by adding sufficient quantity of the dye to alcohol to make a saturated solution. Some undissolved stain at the bottom of the bottle is normal. To these stock solutions alcohol is added from time to time, taking care that the undissolved stain always remains. When required for use add 5 c.c. of the saturated alcoholic solution to 95 c.c. of distilled water, and filter.

Use the solution prepared especially for microscopic work by rubbing.

The watery solutions soon decompose, and must be made only as required for use. Methylene-blue, however, may be made up as a saturated aqueous solution, as it is permanent. Always filter a stain before use.

## 2. Kuhne's Methylene-blue.

Methylene-blue,	1.5 gm. ;
Absolute alcohol,	10 c.c. ;
Carbolic acid (1 : 20),	100 c.c.

Stain films five minutes.

## 3. Löffler's Methylene-blue.

Saturated alcoholic solution of methylene-blue,	30 c.c. ;
Solution of potassium hydrate in water (1 : 10,000),	100 c.c.

## 4. Carbol-thionin-blue.

Thionin-blue,	1 gm. ;
Carbolic acid (1 : 40),	100 c.c.

Dilute 1 volume of the stain with 3 of water, when required for use. Stain from three to five minutes.

## 5. Anilin-water Solution.

Anilin oil,	5 c.c. ;
Water,	100 c.c.

Shake together, allow to stand for five minutes, and filter through a moistened filter.

## 6. Anilin-gentian-violet and Anilin-fuchsin.

Anilin-water,	10 parts;
Saturated alcoholic solution of gentian-violet or fuchsin,	1 part.

**7. Gram's Iodin Solution.**

Iodin,	1 part;
Potassium iodid,	2 parts;
Distilled water,	300 "

**8. Ziehl's Carbol-fuchsin.**

Basic fuchsin,	1 part;
Absolute alcohol,	10 parts;
Carbolic acid (1 : 20),	100 "

**9. Gabbet's Blue.**

Sulphuric acid (25 per cent. solution),	100 c.c.;
Methylene-blue,	2 gm.

Allow the diluted acid to stand twenty-four hours or until it is cold before adding the methylene-blue.

**10. Unna's Polychrome Methylene-blue.**

Methylene-blue,	1 part ;
Potassium carbonate,	1 "
Water,	100 parts.

Must be ripened for months. The ripened solution may be procured from Grüber.

**11. Ehrlich's Triacid Stain.**

Saturated aqueous solution of orange G,	120 parts.
Acid fuchsin,	80 "
Methyl-green,	100 "
Distilled water,	300 "
Absolute alcohol,	180 "
Glycerin,	50 "

Never shake the solution. Pipet from the top what is needed for use.

**12. Ehrlich-Biondi Stain.**

Is best procured ready made from Grübler.

**13. Chenzinsky-Plein Stain.**

Saturated aqueous solution of	
methylene-blue,	40 c. c. ;
Alcoholic (70 per cent.) solu-	
tion of eosin (1 : 200),	20 c. c. ;
Distilled water,	40 c. c.

The best results are obtained by staining for twenty-four hours. Fairly good results may be obtained by using warmed stains for fifteen minutes.

**14. Normal Salt Solution.**

Distilled water,	100 c. c. ;
Sodium chlorid,	0.75 gm.

**15. Acid Alcohol.**

Alcohol (70 per cent.),	97 c. c. ;
Hydrochloric acid,	3 c. c.

**16. Zenker's Fluid.**

Potassium bichromate,	2.5 gm. ;
Sodium sulphate,	1 gm. ;
Corrosive sublimate,	5 gm. ;
Glacial acetic acid,	5 c. c. ;
Water,	<i>ad</i> 100 c. c.

Do not add the acetic acid until ready for use.

Fix tissues from one to twenty-four hours.

Wash in running water from twelve to twenty-four hours. Preserve in 80 per cent. alcohol.

**17. Cleaning Mixture, for Slides, Cover-glasses, and Glassware.**

Potassium bichromate,	6 gm.;
Sulphuric acid,	6 c.c.;
Water,	100 c.c.

Wash in water and alcohol.

**18. Nessler's Solution.**

A. Potassium iodid,	35 gm.;
Water,	200 c.c.
B. Mercuric chlorid,	16 gm.;
Water,	500 c.c.

Add B to A until faint show of excess is indicated, then add 160 grams of solid potassium hydrate. Dilute to 1 liter, and add strong solution of mercuric chlorid, little by little, until the red mercuric iodid just begins to be permanent. Do not filter. Solution should be pale straw-color. It is improved by age.

**19. Phenol-sulphonic Acid.**

Sulphuric acid (pure and concentrated),	148 c.c.;
Distilled water,	12 c.c.;
Pure carbolic acid,	24 gm.

## IV. TABLE OF SYNONYMS.

The scientific names of species used throughout this work are those adopted by Migula. They are preferable because they conform to the laws of scientific nomenclature and priority. The following table gives the synonyms of many of the common species in alphabetic order :

## BACILLUS—

albolactus, Mig.; *B. lactis albus*, Löffler.  
 amylovorus (Burrill), De Toni ; *M. amylovorus*, Burrill.  
 atterimus, L. et N.; *B. mesentericus niger*, Lunt.  
 avium, Mig.; *B. diphtheriæ avium*, Kruse.  
 chauvæi, Arloing, Cornevin et Thomas ; *B. carbonis*,  
 Mig.; *B. anthracis symptomatici*, Kruse.  
 coli, Mig.; *Bact. coli commune*, Escherich.  
 diaphanus, Mig.; *Halibacterium pellucidum*, Fischer.  
 dysenteriæ (Kruse), Mig.; *B. dysenteriæ liquefaciens*,  
 Kruse.  
 equi, Mig.; *B. equi intestinalis*, Dyar et Keith.  
 fischeri, Mig.; *Photobacterium fischeri*, Beyerinck ; *B.*  
*phosphorescens indigenus*, Kruse.  
 globigii, Mig.; *B. mesentericus ruber*, Globig.  
 indicus, Koch ; *B. indicus ruber*, Mig.  
 intricatus, Mig.; *Cladothrix intricata*, Russell.  
 limnophilus, Mig.; *B. limosus*, Russell.  
 mesentericus, Mig.; *B. mesentericus fuscus*, Flügge.  
 mirabilis, Mig.; *Proteus mirabilis*, Hauser.  
 murium, Mig.; *B. typhi murium*, Löffler.  
 niger, Mig.; *B. lactis niger*, Gorini.  
 ozænæ, Mig.; *B. foetidus ozænæ*, Hajek.  
 phosphorescens, Fischer ; *Photobacterium indicum*, Beyerinck ; *B. phosphorescens indicus*, Kruse.



## BACILLUS—

- phosphoricus, Mig.; *B. argenteo-phosphorescens* III., Katz.  
 prodigiosus, Flügge; *Monas prodigiosa*, Ehrenberg; *Bact. prodigiosum*, Schröter; *M. prodigiosus*, Cohn.  
 pseudotuberculosis, Mig.; *B. pseudotuberculosis liquefaciens*, Kruse.  
 suipestifer, Kruse; *B. of hog-cholera*, Salmon-Smith; *B. cholerae suum*, Mig.  
 sulfureus, Mig.; *Proteus sulfureus*, Lindenborn.  
 typhosus, Mig.; *B. typhi abdominalis*, Mig.  
 vulgaris, Mig.; *Proteus vulgaris*, Hauser.  
 vulgatus, Mig.; *B. mesentericus vulgatus*, Flügge.  
 zenkeri, Hauser; *Proteus zenkeri*, Hauser.  
 zopfii, Mig.; *Bact. zopfii*, Kurth.

## BACTERIUM—

- aceti, Zopf; *Ulvina aceti*, Kützing; *Mycoderma aceti*, Thomsen.  
 acidi lactici, Mig.; *B. acidi lactici* I., Hüppe.  
 aërogenes, Mig.; *Bact. lactis aërogenes*, Escherich.  
 amethystinum, Mig.; *B. membranaceus amethystinus*, Eisenberg.  
 anthracis, Mig.; *B. anthracis*, Koch.  
 anthracoides, Mig.; *B. anthracoides*, Hüppe et Wood.  
 aquatile, Mig.; *B. aquatile*, Frankland.  
 arborescens, Mig.; *B. arborescens*, Frankland.  
 aurescens, Mig.; *B. aurescens*, Frankland.  
 bienstockii, Schröter; *B. coprogenes parvus*, Bienstock.  
 bovis, Mig.; *Pneumobacillus liquefaciens bovis*, Arloing; *B. pneumonicus liquefaciens*, Kruse.  
 brunneum, Mig.; *B. brunneus*, Adametz-Wichmann.  
 buccale, Mig.; *Leptothrix buccalis*, Robin.  
 candicans, Mig.; *B. candicans*, Frankland.

**BACTERIUM—**

- capsulatum, Pfeiffer ; *B. capsulatus*, Pfeiffer, Koch.  
carotarum, Mig. ; *B. carotarum*, Koch.  
chlorinum, Mig. ; *B. chlorinus*, Frankland.  
citreum, Mig. ; *B. citreus*, Frankland.  
cœruleum, Mig. ; *B. cœruleus*, Smith.  
columbarum, Mig. ; *B. diphtheriæ columbarum*, Löffler.  
coprogenes, Mig. ; *B. coprogenes fœtidus*, Flügge.  
cuniculicida, Koch ; *B. cholerae gallinarum*, Flügge ;  
    *Bact. septichæmiæ*, Schröter.  
cuniculi, Mig. ; *B. cuniculi pneumonicus*, Kruse.  
endocarditidis, Mig. ; *B. endocarditidis capsulatus*, Weichselbaum.  
felis, Mig. ; *B. felis septicus*, Kruse.  
giardi, Mig. ; *B. phosphorescens giardi*, Kruse.  
keratomalaciæ, Mig. ; *B. septicus keratomalaciæ*, Babes.  
lacteam, Mig. ; *B. lactis III.*, Kruse.  
lacticum, Mig. ; *B. lacticus*, Kruse ; *B. lactis acidi*, Liebmann.  
laerii, Mig. ; *B. viscosus I.*, van Laer ; *B. viscosus cerevisiæ*, Kruse.  
lepræ, Mig. ; *B. lepræ*, Hansen.  
mallei, Mig. ; *B. mallei*, Löffler.  
murisepticum, Mig. ; *B. murisepticus*, Flügge ; *B. murinus*, Schröter.  
mycoides, Mig. ; *B. mycoides roseum*, Scholl-Holschewnikoff.  
nephritidis, Mig. ; *B. nephritidis interstitialis*, Letzerick.  
palubarium, Mig. ; *B. cholerae columbarum*, Kruse.  
phosphorescens, Fischer ; *Photobacterium phosphorescens*, Beyerinck.  
proteus, Mig. ; *Proteus capsulatus septicus*, Banti ; *B. capsulatus septicus*, Kruse.

## BACTERIUM—

pseudodiphthereticum, Mig.; *Corynebacterium pseudodiphthereticum*, L. et N.

pseudoinfluenzæ, Mig.; *B. pseudoinfluenzæ*, Kruse.

pseudotuberculosis, Mig.; *B. pseudotuberculosis*, Pfeiffer;

*Streptobacillus pseudotuberculosis rodentium*, Preisz.

pyocinnabarium, Mig.; *B. pyocinnabarius*, Kruse.

pyogenes, Passet; *B. pyogenes foetidus*, Passet.

rhusiopathiæ, Mig.; *B. rhusiopathiæ suis*, Kitt; *Bact. erysipelatus suis*, Mig.

salivæ, Mig.; *B. salivæ minutissimus*, Kruse.

sempervivum, Mig.; *B. lactis XII.*, Kruse.

smegmatis, Mig.; *B. smegmatis*, Kruse.

subtile, Mig.; *B. subtilis simulans I.*, Bienstock; *B. faecalis I.*, Kruse.

suicida, Mig.; *B. suisepcticus*, Kruse.

termophilum, Mig.; *B. termophilus*, Miquel.

tuberculosis avium, Mig.; *B. tuberculosis avium*, Maffucci; *Mykobacterium tuberculosis avium*, L. et N.

tuberculosis, Mig.; *B. tuberculosis*, Koch; *Mykobacterium tuberculosis*, L. et N.

ubiquitum, Mig.; *B. ubiquitus*, Jordan.

varicosum, Mig.; *B. varicosus conjunctivæ*, Hombert.

vermiculare, Mig.; *B. vermicularis*, Frankland.

vignali, Mig.; *B. g.*, Vignal; *B. buccalis minutus*, Sternberg.

welchii, Mig.; *B. aërogenes capsulatus*, Welch.

xerosis, Mig.; *B. xerosis*, Neisser et Kuschbert.

## MICROCOCCUS—

albocereus, Mig.; *Staphylococcus cereus albus*, Passet.

amplus, Mig.; *M. albicans amplus*, Flügge.

aurantiacus, Cohn; *Staphylococcus cereus aureus*, Mig.

## MICROCoccus—

- aureus, Mig.; *Staphylococcus pyogenes aureus*, Rosenbach; *M. pyogenes aureus*, Mig; *M. pyogenes*, L. et N.  
 cereus, Mig.; *Staphylococcus cereus flavus*, Passet.  
 citreus, Mig.; *Staphylococcus pyogenes citreus*, Passet.  
 citrinus, Mig.; *Diplococcus citreus liquefaciens*, Unna.  
 conglomeratus, Flügge; *M. citreus conglomeratus*, Flügge; *Diplococcus citreus conglomeratus*, Eisenberg.  
 conjunctivitis, Mig.; *M. flavus conjunctivæ*, Gombert.  
 conoideus, Mig.; *Staphylococcus salivarius pyogenes*, Biondi.  
 corrugatus, Mig.; *Merismopedia mesenterica corrugata*, Dyar.  
 coryzæ, Mig.; *Diplococcus coryzæ*, Hajek.  
 cuniculorum, Mig.; *M. pyæmiæ cuniculorum*, Schröter.  
 cyaneus, Cohn; *Bacteridium cyaneum*, Schröter.  
 desidens, Mig.; *M. flavus desidens*, Flügge.  
 faviformis, Mig.; *M. lacteus faviformis*, Flügge.  
 fragilis, Mig.; *Merismopedia fragilis*, Dyar.  
 gonorrhœæ, Flügge; *Gonococcus*, Neisser.  
 intracellularis, Mig.; *Diplococcus intracellularis meningitidis*, Weichselbaum; *Streptococcus intracellularis*, L. et N.  
 lacticus, Mig.; *Sphærococcus acidi lactici*, Marpmann.  
 liquefaciens, Mig.; *M. urææ liquefaciens*, Flügge.  
 luteus, Cohn; *Bacteridium luteum*, Schröter.  
 mollis, Mig.; *Merismopedia mollis*, Dyar.  
 phosphoreus, Cohn; *M. lucens*, v. Tieghem; *M. pflügeri*, Ludwig, ex parte; *Photobacterium phosphorescens*, Beyerinck.  
 pyogenes, Mig.; *Staphylococcus pyogenes albus*, Rosenbach; *M. pyogenes albus*, L. et N.  
 roseus, Flügge; *Diplococcus roseus*, Eisenberg.

## MICROCOCCUS—

- rugatus, Mig.; *M. endocarditidis rugatus*, Weichselbaum.  
 salivarius, Mig.; *Coccus salivarius septicus*, Biondi.  
 stellatus, Frankland; *Coccus stellatus*, Lustig.  
 subflavidus, Mig.; *M. tetragenus subflavus*, v. Besser.  
 tardigradus, Mig.; *M. flavus tardigradus*, Flügge.  
 tardior, Mig.; *Diplococcus flavus liquefaciens tardus*, Unna et Tommasoli.  
 tardissimus, Mig.; *Diplococcus albicans tardissimus*, Flügge.  
 tardus, Mig.; *Diplococcus albicans tardus*, Unna et Tommasoli.  
 tenuis, Mig.; *M. pyogenes tenuis*, Rosenbach.  
 tenuissimus, Mig.; *M. cumulatus tenuis*, v. Besser.  
 varians, Mig.; *Merismopedia flava varians*, Dyar.

## MICROSPIRA—

- annularis, Mig.; *Photobacterium annulare*, Fischer.  
 canalis, Mig.; *Vibrio saprophiles* γ, Weibel.  
 caraibica, Mig.; *Photobacterium caraibicum*, Fischer.  
 comma, Schröter; *Spirillum cholerae asiaticæ*, Flügge;  
     *Vibrio cholerae asiaticæ*, Mig.; *Vibrio comma*, Mig.  
 coronata, Mig.; *Photobacterium coronatum*, Fischer.  
 degenerans, Mig.; *Photobacterium degenerans*, Fischer.  
 delgadensis, Mig.; *Photobacterium delgadense*, Fischer.  
 dunbari, Mig.; *Vibrio dunbari*, Mig.  
 finkleri, Schröter; *Spirillum finkleri*, Mig.; *Vibrio finkleri*, Mig.; *Vibrio proteus*, Mig.  
 glutinosa, Mig.; *Photobacterium glutinosum*, Fischer.  
 luminosa, Mig.; *Photobacterium luminosum*, Fischer.  
 marina, Mig.; *Spirillum marinum*, Russell.  
 metschnikovi, Mig.; *Vibrio metschnikovi*, Gamaleïa.  
 papillaris, Mig.; *Photobacterium papillare*, Fischer.

## MICROSPIRA—

saprophiles, Mig.; *Vibrio saprophiles*,  $\beta$ , Weibel.  
 tonsillaris, Mig.; *Vibrio tonsillaris*, Klein.  
 tuberosa, Mig.; *Photobacterium tuberosum*, Fischer.

## PSEUDOMONAS—

æruginea, Mig.; *Bact. æruginosum*, Schröter; *B. æruginosus*, Schröter; *B. pyocyaneus*, Gessard.  
 alba, Mig.; *B. fluorescens albus*, Zimmermann; *B. fluorescens non-liquefaciens* (?), Eisenberg.  
 eisenbergii, Mig.; *B. fluorescens non-liquefaciens*, Eisenberg  
 europæa, Mig.; *Nitrosomonas europæa*, Winogradsky.  
 fluorescens, Mig.; *B. fluorescens liquefaciens*, Flügge.  
 ianthina, Mig.; *Bact. ianthinum*, Zopf; *B. janthinus*, Zimmermann.  
 javanica, Mig.; *Photobacterium javanense*, Eijkmann.  
 javaniensis, Mig.; *Nitrosomonas javaniensis*, Winogradsky.  
 laurentia, Mig.; *B. violaceus laurentius*, Jordan.  
 litoralis, Russell; *B. litoralis*, Russell.  
 minutissima, Mig.; *B. fluorescens liquefaciens minutissimus*, Unna et Tommasoli.  
 pseudianthina, Mig.; *B. violaceus*, Frankland.  
 tenuis, Mig.; *B. fluorescens tenuis*, Zimmermann.

## SARCINA—

ventriculi, Goodsir; *Merismopedia goodsiri*, Husem;  
*Merismopedia ventriculi*, Robin; *Sarcina fuscescens*, de Bary.

## SPIROSOMA—

attenuatum, Mig.; *Spirillum attenuatum*, Warming.  
 flavescens, Mig.; *Vibrio flavescens*, Weibel.

## SPIROSOMA—

flavum, Mig.; *Vibrio flavus*, Weibel.  
gregarium, Mig.; *Myconostoc gregarium*, Cohn.

## STREPTOCOCCUS—

cerasinus, Mig.; *M. cerasinus siccus*, List.  
citreus, Mig.; *M. citreus*, List-Eisenberg.  
cystitidis, Mig.; *Diplococcus ureæ pyogenes*, Rovsing.  
equi, Schütz; *S. coryzæ contagiosæ equorum*, Eisenberg.  
giganteus, Mig.; *S. giganteus urethræ*, Lustgarten et Mannaberg.  
gracilis, Mig.; *S. coli gracilis*, Escherich.  
mastitidis, Guillebeau; *S. mastitidis sporadicæ*, Guillebeau; *S. agalactiæ contagiosæ*, Kitt.  
pyogenes, Rosenbach; *S. erysipelatos*, Rosenbach; *S. conglomeratus*, Kurth; *S. brevis*, v. Lingelsheim; *S. longus*, v. Lingelsheim; *S. murisepticus*, v. Lingelsheim; *S. septo-pyæmicus*, Biondi.  
septicus, Mig.; *S. septicus liquefians*, Babes; *S. septicus liquefaciens*, Babes.

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